University of Alberta

Regulation Of The Matrix Metalloproteinase-9 Gene: Role Of Nitric Oxide And The Transcriptional Repressor Wilms Tumor 1

by

Marcelo Marcet Palacios ©

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Experimental Medicine

Department of Medicine

Edmonton, Alberta
Spring 2007

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-29708-7
Our file Notre référence
ISBN: 978-0-494-29708-7

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

Dedication

Me da un gran placer dedicarle este trabajo a mi mamá y a mi papá, Dra. Miriam Palacios-Callender y Dr. Marcelo Marcet Sanchez. Este trabajo representa el fruto de una larga carrera estudiantil, la cual hubiera sido imposible sin el apoyo incondicional de mis padres. Vivo orgulloso de ambos y son la fuente de inspiración de mis esfuerzos.

Le voy a dedicar este trabajo también a mis abuelos del alma Dr. Marcelo Enrique Marcet Beato y Dra. Mercedes Sanchez Torres. En especial a mi abuelito Juan Bautista Palacios Beltrán y a mi abuelita Marta Álvarez Roberti. Siempre los tendré presente.

It gives me great pleasure to dedicate this thesis to my mom and dad, Drs. Miriam Palacios-Callender and Marcelo Marcet Sanchez. This thesis would not have been possible without their whole-hearted support. I am exceptionally proud of them; they are my source of inspiration.

I will also dedicate this thesis to my grant parents Dr. Marcelo Enrique Marcet Beato y Dra. Mercedes Sanchez Torres. Especially to my grandfather Juan Bautista Palacios Beltran and my grandmother Marta Alvarez Roberti. You will always be with me.

Abstract

Matrix metalloproteinase-9 (MMP-9) is released by many structural cells such as human lung epithelial cells (LEC) and vascular smooth muscle (VSM) in conditions such as asthma and septic shock. Expression of MMP-9 correlates with the severity of these disorders. Transcriptional regulation of this enzyme, however, is poorly understood.

In a pilot study, rat aortic VSM cells stimulated with a cocktail containing bacterial LPS, IFN-γ, and PMA were shown to express inducible nitric oxide synthase (iNOS) and matrix metalloproteinase-9 (MMP-9). L-NAME, a NOS inhibitor, exerted an inhibitory effect on MMP-9 mRNA levels. NO-mediated upregulation of MMP-9 was cGMP-dependent since ODQ, an inhibitor of sGC, blocked the increased expression of MMP-9, an effect reversed by 8-bromo-cGMP, a soluble analog of cGMP.

To further dissect this pathway the human MMP-9 promoter was studied. A phylogenetic analysis revealed a highly conserved sequence in the 5' flanking region of the MMP-9 gene containing binding sites for the transcription factor Wilms tumor 1 (WT1). We confirmed the presence of WT1 in human lung epithelial cells (LEC) and that treatment with TNF or a cocktail containing LPS, PMA and IFNγ resulted in translocation of WT1 from the nucleus to the cytosol. This translocation coincided with increased expression of MMP-9 and could be blocked by inhibitors of the NO/sGC pathway. WT1 knock down using small interfering RNA upregulated MMP-

9 expression, even in the presence of the NOS inhibitor 1400W. Using chromatin immunoprecipitation we confirmed WT1 binding to the MMP-9 promoter. These findings indicate WT1 is a repressor of MMP-9 regulated by a NO-mediated pathway.

NO regulates lymphocyte proliferation however the mechanism by which this regulation takes place is not known. Because WT1 is regulated by NO and because WT1 dysfunction results in proliferative aberrations it is possible that WT1 mediates NO-dependent regulation of lymphocyte proliferation. WT1 expression in human lymphocytes was confirmed. Exogenously added NO using SNOG reduced the expression of WT1 at the mRNA and protein levels.

The discovery that NO through the regulation of WT1 function controls MMP-9 expression and potentially lymphocyte proliferation adds another piece to the puzzle in mechanisms underling inflammatory processes.

Preface

In this thesis I present one publication (Chapter 2) and two additional works in progress (Chapters 3 and 4) under the general theme of inflammatory reactions. Inflammatory conditions involve many different cell types and mediators. The numerous mediators fall into several categories, including: cytokines, chemokines, enzymes, etc and can all play important roles. I will focus on the role that inducible nitric oxide synthase (iNOS) plays in the regulation of expression and activity of matrix metalloproteinase-9 (MMP-9).

Chapter 1 is introductory. I describe two disorders namely asthma and septic shock as examples of inflammatory conditions that are abundant worldwide; yet there is no cure. These two disorders are models for discussion of common mediators among which iNOS, MMP-9 and cyclooxygenase-2 (COX-2) will be emphasized. One important point is that these mediators are not only made by inflammatory cells (leukocytes) but also by resident or parenchymal cells like vascular smooth muscle (VSM) and lung epithelial cells (LEC). This background information is synthesized into a conceptual model that was used to generate the hypotheses that gave rise to the aims of this thesis.

In chapter 2, the first publication is presented. Using a septic shock model, rat VSM cells were stimulated with a cocktail of proinflammatory mediators. Upon stimulation these cells produced iNOS, MMP-9 and COX-2. We observed that MMP-9 expression required nitric oxide (NO) production and from this point the mechanisms

by which NO regulates MMP-9 became my major focus of research. In the work we concluded that the NO-dependent upregulation of MMP-9 was mediated through soluble guanylate cyclase (sGC) and cyclic GMP (cGMP).

Chapter 3 describes a major extension of this research. These studies were performed in human LEC, using a less aggressive and more physiologically relevant stimulus, TNF. We used a different cell type because these cells are also structural cells that co-upregulate iNOS, COX-2 and MMP-9 in inflammatory conditions. Confirmation of the presence of the pathways described in chapter 2 in another cell type would help elucidate the relevance of these mechanisms and determine if the pathways exist in human cells.

To elucidate downstream targets from cGMP, we hypothesised that PKG and PKA participated in transducing this signal and tested the role of a transcriptional repressor called Wilms tumor 1 (WT1). We concluded that cGMP-dependent activation of PKA regulated the phosphorylation and consequently the translocation of WT1 to the cytosol. This process resulted in de-repression of the MMP-9 promoter and its subsequent TNF-dependent activation.

The novel observation that WT1 participated in the regulation of MMP-9 in a NO-regulated pathway led us to search for WT1 expression in other cell types. In chapter 4 we will describe our findings of the expression of WT1 in lymphocytes and how NO regulates WT1 levels in correlation with lymphocyte proliferation.

In the last chapter, I summarize the major findings of these studies and provide a conceptual model to explain our observations. I integrate these findings with relevant literature, discuss their relevance and outline future research directions essential to advance this field.

Acknowledgment

This thesis has been brought about by the work of a large number of excellent collaborators but it is also the product of the influence of great teachers whom I had the good fortune to have.

I would like to acknowledge the teachers and tutors from Cuba, England and Canada who provided me support and education along the way to this point. My most memorable tutors and teachers because of the impact that they had in my life and their outstanding dedication include: Prof. Lázaro Cárdenas Vasquez from the Polytechnic Institute Mártires de Girón, Cuba; Prof. Jed Armstrong from Lewisham College, United Kingdom. Dr. A. Dean Befus, from the University of Alberta, Canada.

Among my collaborators the members of the Pulmonary Research Group have played an instrumental role. Dr. Marina Ulanova was a significant source of motivation and knowledge and I thank her for her help and enthusiasm, for been a great teacher and a fantastic collaborator. I acknowledge the role of my colleague and co-Ph.D. student Dr. Derrick Gibbings. The excellent support provided by Rene Dery, Chris Laurent, Melisa Steward, Melanie Abel and Lynelle Watt.

I would like to acknowledge the help and support provided by the members of my supervisory committee, Dr. Allan Murray, Dr. Marek Duszyk, Dr. Marek Radomski

and my supervisors Dr. Irvin Mayers, and Dr. A. Dean Befus. It has been an honour and a privilege to work under the direct supervision of Dean. Thank you for been an exceptional supervisor and thank your for your patience and friendship.

I would like to thank my family especially Dr. Miriam Palacios-Callender (mom) and Alec Callender, Dr. Marcelo E. Marcet Sanchez (dad) and my loving sister Dr. Miriam Marcet Palacios, all of which have always been there for me without who this endeavour would not have been possible.

Table of Contents

Chapter I: Introduction 1

1. Inflammatory diseases and their relevance 1

A. Introduction to inflammation 1

B. Septic shock 2

i. Vascular smooth muscle mediators in septic shock 3

- Vascular smooth muscle in health 3
- Vascular smooth muscle in septic shock 5
- Inducible nitric oxide synthase role in septic shock 7
- Matrix metalloproteinase-9 role in septic shock 8

C. Asthma 9

i. Lung epithelial cells and their mediators in asthma 10

- Physiology of lung epithelial cells 10
- Lung epithelial cells: Role in asthma 15
- Nitric oxide: Role in asthma 17
- Matrix metalloproteinase-9: Role in asthma 20

2. Cyclooxygenase-2 21

A. The cyclooxygenase family 21

B. Structure of cyclooxygenase 22

D. Cyclooxygenase-2: role in asthma and septic shock 23

3. Matrix metalloproteinase-9 24

A. The matrix metalloproteinase family 24

i. Regulation of matrix metalloproteinases 25

C. Matrix metalloproteinase-9 structure and regulation 26

i. Gene and protein structure 26

ii. Regulation 26

- Proteolytic activation 26
- Transcriptional regulation of MMP-9 27
- Regulation by nitric oxide and peroxynitrite 31

iii. Posttranscriptional fate of MMP-9 in the lung 35

- Expression and activity 35
- Degradation of MMP-9 35

4. Inducible nitric oxide synthase 36

A. The nitric oxide synthase family 36

B. Inducible nitric oxide synthase structure and regulation 37

i. Protein structure 37

ii. Regulation 38

C. Nitric oxide chemistry 39

5. Wilms tumor 1 40

A. Wilms tumor: An introduction 40

B. Wilms tumor 1 structure and regulation 42

i. Gene and protein structure 42

ii. Protein-protein interactions 45

iii. Protein-RNA interactions 48

iv. WT1 chemical modifications 49
v. WT1 DNA binding sites 50
vi. WT1 gene targets 50
vii. WT1 expression 52
6. Synthesis and conceptual model 52
7. Hypothesis 56
8. Objectives 56
8. References: 57
Chapter II: NO and cGMP upregulate MMP-9 in vascular smooth muscle 89
1. Title 89
2. Introduction 89
3. Materials and methods 91
4. Results 96
5. Discussion 109
6. Tables 113
7. References 115
Chapter III: WT1 regulates MMP-9 via nitric oxide 121
1. Title 121
2. Introduction 121
3. Materials and methods 123
4. Results 134
5. Discussion 155
6. References 160

Chapter IV: WT1 mediates nitric oxide-dependent repression of lymphocyte proliferation 167

1. Title 167

2. Introduction 167

3. Materials and methods 168

4. Results 172

5. Discussion 178

6. References 179

Chapter V: General Discussion and Conclusions 182

1. Summary of major findings 182

2. Conceptual model 184

3. Results and their relation to original aims/questions 185

4. Results and their relation to the literature 188

5. Relevance/significance of findings 190

6. Future directions 191

7. References 193

Appendix 1 194

Appendix 2 195

Appendix 3 196

Appendix 4 197

Appendix 5 199

Appendix 6 200

List of tables

Table 1.1: WT1 DNA binding sites from Menke A.L. *et al.*. 51

Table 2.1: Summary of RT-PCR primer details. 113

Table 2.2: Summary of real time PCR primer and probe sequences and accession numbers..... 114

List of figures*

Figure 1.1: Role of vascular smooth muscle in the pathogenesis of septic shock.. 6

Figure 1.2: Common human lung epithelial cells and their location within the airways.. 11

Figure 1.3: Role of alveolar type II (ATII) cells in inflammation. 16

Figure 1.4: Plasmin and MMP involved in the proteolytic activation of MMP-9. 27

Figure 1.5: Functional transcription factor binding sites in the MMP-9 promoter..... 28

Figure 1.6: Summary of regulatory pathways involved in MMP-9 gene expression. 30

Figure 1.7: Mechanisms through which NO regulates MMP-9 enzyme activity. 32

Figure 1.8: Dual role of NO in MMP-9 regulation. 34

Figure 1.9: Domain structure and cofactors of the nitric oxide synthase family.. 38

Figure 1.10: Wilms tumor 1 (WT1) gene structure and protein domains..... 42

Figure 1.11: WT1 major molecular weight (MW) alternative isoforms..... 44

Figure 1.12: WT1 regulatory mechanisms..... 45

Figure 1.13: Conceptual Model. 55

Figure 2.1: Fluorescence detection of intracellular NO production. 97

Figure 2.2: Characterization of the septic shock model at the transcriptional level. 98

Figure 2.3: Characterization of the septic shock model at the protein level..... 100

Figure 2.4: MMP-9, iNOS, and COX-2 enzymatic activity in A7r5 cells..... 102

Figure 2.5: Effect of L-NAME treatment on iNOS, COX-2, and MMP-9 mRNA.. 104

Figure 2.6: MMP-9 gene induction is not mediated by $ONOO^-$. 106

Figure 2.7: NO up-regulates MMP-9 via a sGC-dependent pathway..... 108

Figure 3.1: NO regulates MMP-9 gene expression in human LEC A549. 137

Figure 3.2: MMP-9 gene expression is regulated by sGC and PKA. 139

Figure 3.3: Conservation of MMP-9 across multiple species..... 141

Figure 3.4: Human lung epithelial cells express WT1..... 143

Figure 3.5: WT1 is expressed in the nuclei of human lung epithelial cells.. 145

Figure 3.6: TNF does not affect WT1 mRNA and protein expression.. 147

Figure 3.7: TNF induces WT1 translocation from the nucleus to the cytosol.. 149

Figure 3.8: WT1 functions as a MMP-9 repressor... 152

Figure 4.1: Flow cytometry of peripheral blood leucocytes showed expression of WT1 in lymphocytes and monocytes but not in granulocytes.. 173

Figure 4.2: PHA-stimulated proliferating T cells express increased levels of WT1 175

Figure 4.3: NO inhibits PHA-induced upregulation of WT1.. 177

Figure 5.1: Thesis conceptual model. 184

*All figures are original

Abbreviations

AC, adenylate cyclase

cAMP, cyclic adenosine monophosphate

CFTR, cystic fibrosis transmembrane conductance regulator

cGMP, cyclic guanosine monophosphate

COX-2, cyclooxygenase-2;

DAF-FM, diaminofluorescein-fluorometry;

Dex, dexamethasone;

eNOS, endothelial nitric oxide synthase or NOS-III

GAPDH, glyceraldehyde-3-phosphate dehydrogenase;

IFN-γ, interferon-gamma;

iNOS, inducible nitric oxide synthase or NOS-II

LEC, lung epithelial cells;

L-NAME, N^{ω}-nitro-L-arginine methyl ester;

LPS, lipopolysaccharide;

MMP, matrix metalloproteinase;

MnTMPyP, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride;

nNOS, neuronal nitric oxide synthase or NOS-I

NO, nitric oxide;

ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one;

PDE, Phosphodiesterase

PGE_2, prostaglandin E_2;

PKA, cAMP-dependent protein kinase.

PKG, cGMP-dependent protein kinase;

PMA, phorbol 12-myristate 13-acetate;

RT-PCR, reverse transcription-polymerase chain reaction;

sGC, soluble guanylate cyclase

SOD, superoxide dismutase;

Syk, Spleen tyrosine kinase

VSM, vascular smooth muscle;

WT1, Wilms tumor 1.

Chapter I: Introduction

1. Inflammatory diseases and their relevance

A. Introduction to inflammation

Many medical books define inflammation by the classic signs of pain, heat, redness and swelling resulting in loss of function. Further, at the cellular level inflammation involves a complex sequence of processes, including dilatation of arterioles, capillaries and venules with increased permeability and blood flow, exudation of fluids including plasma proteins and leukocyte migration into the injured tissue.

But what really goes on at the molecular level? Inflammation is produced by any injury or damage to the body such as mechanical trauma, toxins and neoplasia, and commonly occurs as a response to invasion of the host by foreign microbes. Leukocytes play a critical role in inflammatory processes and their accumulation and activation at the site of injury is critical in the pathogenesis of inflammatory conditions. Eosinophils, neutrophils, mast cells and macrophages, in response to chemokines migrate to sites of injury, become activated and release mediators.

Although leukocytes are critical mediators of inflammation and are capable of producing iNOS, MMP-9 and COX-2, other structural cell types also contribute in a significant way to release of these mediators. In the following section, two inflammatory conditions, namely septic shock and asthma will be described. Although we recognize the relative importance of leukocytes in these disorders, we

will focus on the pathogenic roles of VSM and LEC and emphasize the need for better understanding of the pathogenesis of these conditions.

B. Septic shock

Although inflammation is set in place to stop further injury and to repair damage, this process can in some instances overshoot and produce more damage than that originally inflicted by the pathogen. Inflammation-induced damage can generate more inflammation creating a vicious cycle where the patient can worsen quickly. Septic shock is a perfect example of what happens when inflammation goes too far, as there is substantial evidence that it occurs as a result of an exaggerated host response to microorganisms (Fig. 1.1).

Septic shock is characterized by hypotension and vascular collapse, resulting in multiple organ failure and death. The most common cause of sepsis is through the contamination of the blood with bacteria. Sepsis refers to the systemic responses induced by infection and may result in severe systemic hypotension. Septic shock is common and about half of the patients with sepsis may go into shock with 40-60% mortality. It is estimated that 215,000 deaths occur in North America every year from sepsis. When a patient develops shock the worsening circulatory system fails to provide sufficient blood and oxygen to vital organs. Symptoms include a severe fall in blood pressure and hyporeactivity to vasoconstrictor agents leading to dysfunction of major organs like the brain, liver and lungs resulting in death. There is no successful treatment for septic shock highlighting the need for a better understanding of this condition.

The most common cause of septic shock is through gram-negative infections, however gram-positive induced septic shock is also observed. Virus, fungi and parasites account for a low proportion of septic shock cases. Bacterial lipopolysaccharide (LPS) is the major endotoxin responsible for the high morbidity and mortality of gram-negative septic shock. The presence of bacterial products in blood induces a number of cytokines such as TNF, IL-1β, IFN-γ and IL-6. These cytokines, in combination with bacterial products induce production of mediators such as MMP-9, iNOS and COX-2 from VSM and other cells (Fig. 1.1).

i. Vascular smooth muscle mediators in septic shock

MMP-9 and iNOS are important mediators involved in the pathogenesis of septic shock. A major source of these mediators is VSM. In the following section, I will describe the role of VSM in health and how these mediators (MMP-9 and iNOS) released from VSM contribute to the pathogenesis of septic shock.

- ***Vascular smooth muscle in health***

The main role of smooth muscle is to perform a number of structural movements. It is named smooth muscle because in contrast to skeletal muscle, its fibres lack striations. VSM cells are arranged in helical or circular layers around larger blood vessels and in a single circular layer around arterioles. Smooth muscle performs housekeeping tasks such as moving food along the intestine, controlling pupil size, and controlling peripheral vascular resistance.

VSM is responsible for the distribution of blood flow throughout the body. It controls peripheral resistance, arterial and venous tone. The contractile mechanisms of VSM

are different to those of skeletal and cardiac muscle. VSM lacks the regulatory protein troponin, and actin and myosin are not arranged into distinct bands. Contraction is initiated by electrical, chemical or mechanical stimuli. Electrical depolarization of VSM may result in Ca^{2+} influx into the cells resulting in a contractile response. Additionally, many chemical stimuli including norepinephrine, vasopressin, endothelin-1, angiotensin II and thromboxane A2 can lead to contraction of VSM. These chemical signals act through specific receptors and independent transduction pathways, but the end result is to increase Ca^{2+} influx from intracellular and extracellular storages. Free Ca^{2+} binds to calmodulin which then activates myosin light chain kinase (MLCK), the enzyme responsible for the activation of myosin light chain (MLC). This leads to cross-bridge formation between myosin heads and actin filaments resulting in VSM contraction.

There are three major mechanisms to control contraction. First, the phosphatidylinositol pathway can be activated by norepinephrine (via α1-adrenoceptors), angiotensin II (via AII receptor) and endothelin I (via ET_A receptors). These receptors activate phospholipase C (PLC) which will catalyze the formation of inositol triphosphate (IP3), which in turn stimulates the release of Ca^{2+} from the sarcoplasmic reticulum. PLC will also produce diacylglycerol (DAG), activating protein kinase C (PKC) which can mediate VSM contraction in a Ca^{2+}-independent manner. Secondly, the adenylate cyclase (AC) pathway can be activated by β2-adrenoceptors. This receptor is activated by epinephrine resulting in the formation of cyclic AMP (cAMP) that can inhibit MLCK thus leading to relaxation as a result of MLC inactivity. The third regulatory pathway is the nitric oxide (NO) pathway.

Endothelium-derived NO reaches VSM cells through diffusion and activates soluble guanylate cyclase (sGC). This enzyme catalyzes the formation of cyclic GMP (cGMP), resulting in the activation of cAMP-dependent protein kinase (PKG). PKG phosphorylates different ion channels and pumps, resulting in a reduction of cytosolic Ca^{2+}.

- ***Vascular smooth muscle in septic shock***

VSM can produce reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and reactive nitrogen species like NO and peroxynitrite ($ONOO^-$). Cell membrane-associated NADPH-oxidases are responsible for the production of ROS, whereas inducible nitric oxide synthase (iNOS) is involved in the generation of NO and $ONOO^-$. Additionally, neutrophils also produce high levels of O_2^-, which in combination with the VSM-derived O_2^- reacts with NO to generate $ONOO^-$. $ONOO^-$ and NO are major mediators generated by VSM that result in tissue damage in shock conditions.

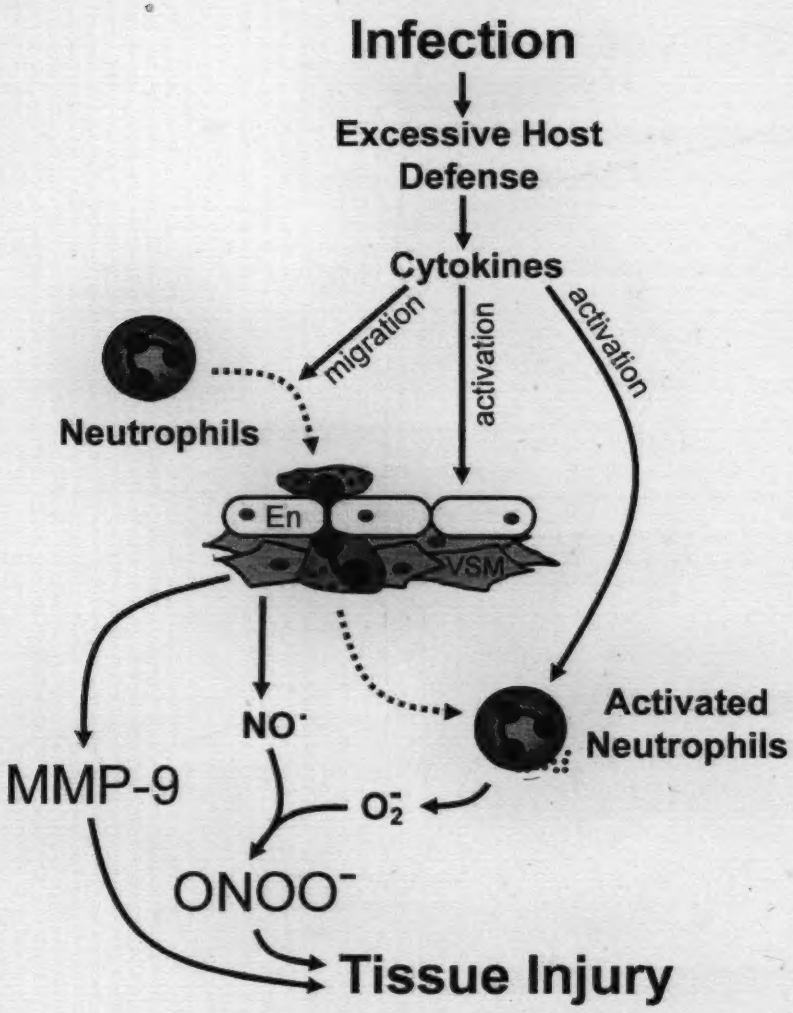


Figure 1.1: Role of vascular smooth muscle (VSM) in the pathogenesis of septic shock. Upon infection host defence is over-amplified resulting in the production of cytokines that stimulate leukocyte (e.g. neutrophils) migration to tissue. Endothelial (En) cells and VSM are stimulated (activation) by cytokines and bacterial LPS resulting in the over expression of iNOS and MMP-9. High NO levels react with superoxide (O2-) forming peroxynitrite (ONOO-). Collectively, MMP-9 and ONOO- contribute to tissue damage which may result in multiple organ failure and death.

- ***Inducible nitric oxide synthase role in septic shock***

Cytokines and bacterial products signal the upregulation of iNOS in many tissues. Overexpression of iNOS and over-production of NO in septic shock are responsible for vasodilatation and tissue damage. Administration of the NOS inhibitor N^{ω}-methyl-L-arginine (L-NMMA) in septic shock patients restores blood pressure and reverses the hyporesponsiveness to vasoconstrictor agents (1). In rats, pretreatment with dexamethasone, a transcriptional inhibitor of iNOS, prevents LPS-induced iNOS expression and attenuates circulatory failure and hyporeactivity to vasoconstrictor agents (2). During shock, iNOS mRNA and protein are upregulated in a number of tissues including heart, pancreas, kidney, liver, aorta, VSM cells, and lung (3-5). Although high levels of NO may enhance body defence against infectious agents, excessive induction of iNOS in these tissues and in particular the vasculature may cause shock, leading to tissue damage and organ failure (6). Administration of anti-TNF prevents the onset of septic shock. Anti-TNF or TNF-receptor antagonists reduce pulmonary iNOS expression with similar observations with anti-IFN-γ antibody treatment (7). The pathways by which TNF, LPS and IFN-γ induce iNOS expression are similar to those described below for MMP-9.

In addition to vasodilatory effects of NO in shock patients, it may also contribute to the formation of $ONOO^-$ or other ROS (8) leading to endothelial damage and increased systemic micro-vascular permeability. NO and $ONOO^-$ have a direct effect on VSM leading to reduced contractility through energy and Ca^{2+} depletion (1).

- ***Matrix metalloproteinase-9 role in septic shock***

MMP-9 is produced constitutively at low to undetectable levels throughout the body. In the presence of the appropriate triggers, MMP-9 is upregulated. *In vitro* LPS has been demonstrated to induce the expression of MMP-9 in neutrophils and monocytes (9, 10). Injection of LPS in humans results in a significant increase of levels of MMP-9 in plasma (11). Additionally, MMP-9 levels are elevated in sepsis and septic shock patients and these levels correlated with severity and mortality (12, 13). It has been suggested that a selective MMP-9 inhibitor could have therapeutic value since MMP-9 deficiency in mice has a protective effect in an endotoxemia model (14). However, contrasting data has shown that MMP-9 blockade might be harmful in the treatment of sepsis (15).

Although determining the role of MMP-9 in sepsis and septic shock has been challenging it has been postulated that MMP-9 could play a role in facilitating the recruitment of leukocytes to the site of inflammation (16). MMP-9 has high affinity for degraded collagen (gelatin) and type IV collagen, both compounds present in basement membranes. Thus MMP-9 is thought to facilitate leukocyte transmigration through degradation of the extracellular matrix. Neutrophils in particular are regarded as an important source of MMP-9 in the early host defence against bacterial infection. In fact in MMP-9 deficient mice neutrophils have a reduced capacity to migrate to the site of infection (15). In conjunction with neutrophil-derived MMP-9 release, macrophages produce TNF converting enzyme (TACE) and pro-TNF (17), which results in the formation of TNF. TNF is important in the stimulation of MMP-9

production by many structural cells (see later), promoting *de novo* production of MMP-9 at the site of injury.

C. Asthma

Asthma is a disorder of the airways that has intrigued researchers for decades. Over 100 years ago, asthma was described as an abnormality of airway irritability (18). Today, the definition of asthma is more complex. The Canadian Asthma Consensus Guidelines defines it as a disorder of the airways characterized by paroxysmal or persistent symptoms (dyspnea, chest tightness, wheeze and cough), with variable airflow limitation and airway hyperresponsiveness to a variety of stimuli. Airway inflammation (including mast cells and eosinophils) or its consequences are important in the pathogenesis and persistence of asthma. Although mortality rates have fallen since 1990, asthma can be fatal killing about 290 Canadians and affecting more than 2.7 million Canadians every year.

As the definition states, consistent findings in lungs from asthmatics include increased cells in the lamina propria (mainly eosinophils and mast cells), smooth muscle and mucous glands are increased in bulk, thickening of basement membrane, infiltration of the epithelium with inflammatory cells and shedding of the epithelium (19).

Epithelial shedding or desquamation is induced by eosinophil-derived substances such as cationic proteins and elastase. Although epithelial shedding is not specific to asthma, it is thought to account for asthma symptoms since inhaled corticosteroids result in epithelium repair before AHR improvements are seen (20). Understanding the role of epithelial cells in asthma is a challenging task. In the next part our current

understanding of the role of the lung epithelium will be discussed with an emphasis on the role of epithelial cells during inflammation.

i. Lung epithelial cells and their mediators in asthma

- ***Physiology of lung epithelial cells***

Gas exchange takes place in the lungs. Due to their intrinsic exposure to the environment they have evolved highly sophisticated defence systems to protect against fungal, parasitic, bacterial and viral infections. Central to these mechanisms, the lung epithelium provides the first line of defence against invading pathogens, allergens and pollutants. The epithelium consists of numerous cell types with specific functions. The cellular complexity of the lungs includes over 40 cell types, of which at least 12 are found in the epithelium (21). The physiological role of each epithelial cell type is linked to their location in the airways. The three major regions of the airways are: cartilaginous bronchi, membranous bronchioles and gas exchange ducts (22) (Fig. 1.2).

The cartilaginous bronchi display ciliated, **columnar epithelial cells.** The cilia are surrounded by smaller microvilli on each cell. These cilia move the superficial liquid (mucous) towards the pharynx. The mucous is biphasic with an aqueous sol layer or periciliary layer where cilia beat, and a more apical gel layer over the tips of the cilia. It is thought that surface epithelial cell secretions create the sol layer, whereas submucosal glands release the denser gel layer.

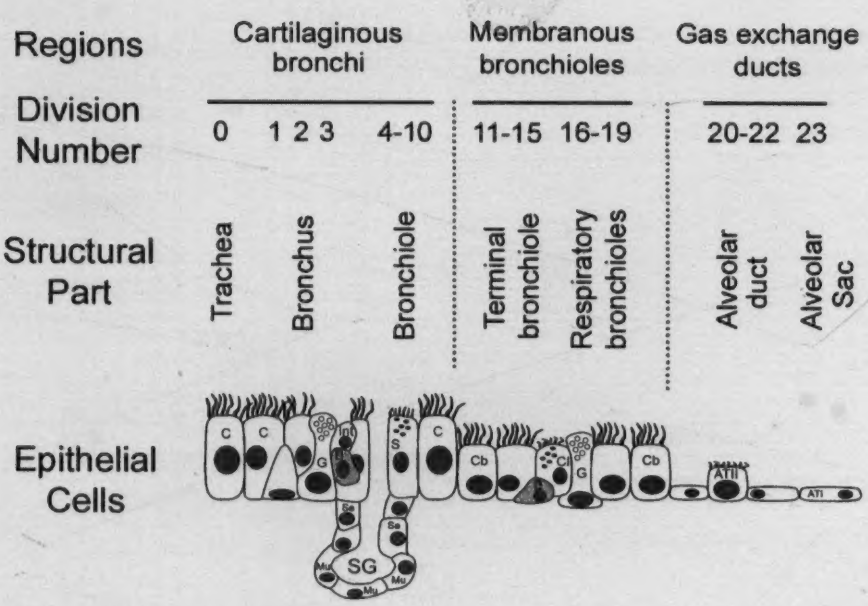


Figure 1.2: Common human lung epithelial cells and their location within the airways. Columnar epithelial cells (C), goblet cells (G), basal cells (B), intermediate cells (Int), surface serous epithelial cells (S), mucous epithelial cells (Mu), submucosal serous epithelial cells (Se), cuboidal epithelial cells (Cb), Clara cells (Cl), alveolar epithelial type I cells (ATI) and alveolar epithelial type II (ATII). ATII do not have cilia but display smaller microvilli. Mu and Se are present in the submucosal glands (SG).

The cartilaginous bronchi display submucosal (lamina propria) glands, also unique to this region. Within these glands we find two additional epithelial cell types, namely the **submucosal serous epithelial cells** (lie upstream as part of the channel) and **mucous epithelial cells** (found downstream). There are about 4000 glands in the

trachea alone. These glands secrete mucins, water and electrolytes into the lumen of the airways. Serous discharge is aided by contractile **myoepithelial cells**, which form a basket around the gland. Although **goblet cells** also secrete these components, they are found also in lower airways disappearing towards the terminal bronchioles. We have approximately 6800 goblet cells per mm^2 of epithelial surface, appearing in a ratio of about 1 goblet cell per 5 ciliated epithelial cells (23), however goblet cell density decreases in the membranous bronchioles. Other epithelial cells called **basal cells** are found along the basal lamina hence the name, and are thought to be precursor cells for other epithelial cell types. **Surface epithelial serous cells** are also found in this region. These cells are located in the epithelial surface and they are different from submucosal serous epithelial cells, which are found within submucosal glands. **Brush cells** are scarce and found mainly in the cartilaginous bronchi (not shown). Brush cells are present in the basement membrane and have characteristic filament bundles throughout their cytoplasm and have a dense population of microvilli on the luminal surface. Brush cells make only about 1% of the cells found in the tracheal epithelium, the site where they are most numerous (24), but their function is unknown. **Intermediate cells**, usually present above the basal cells and are suspected to be differentiating cells as their name implies.

The membranous bronchioles comprise the terminal bronchioles and respiratory bronchioles. Ciliated epithelial cells are also present but due to their smaller size are called **cuboidal epithelial cells**. In this region the epithelium is partially ciliated because cuboidal epithelial cells are intercalated with domed cells that lack cilia, called **Clara cells**. Clara cells are restricted to the terminal bronchioles and have

prominent membrane bound granules and are a source of apoproteins, lipoproteins, and glycoproteins. During tissue injury Clara cells function as progenitor cells to ciliated cells.

Interestingly, the transition from the respiratory bronchioles to the gas exchange ducts is abrupt with the replacement of cuboidal epithelial cells with **squamous epithelial cells** lacking cilia. Fortunately, another abrupt change is the disappearance of goblet cells, thus minimizing mucous secretion in the unciliated epithelial region and preventing backflow of mucus and its stasis.

Alveolar type II (ATII) and **alveolar type I epithelial cells** (ATI) are the only epithelial cells found within the alveoli. The key role of these two cell types is to participate in gas exchange. Type I cells cover 96% of the peripheral lung area, whereas type II cells cover the remaining 4%, in total equivalent to 70 m^2 (25). These two cell types were defined in 1952 with electron micrograph images (26). As recently as 1988 ATI were thought to be merely flatter cells to minimize the distance between the alveolar air space and the pulmonary capillaries (27). In addition to their morphology, the molecular phenotype can differentiate ATI and ATII cells. We now know that ATI can not divide and are derived from ATII cells through a tightly controlled process (28). Differentiated ATI cells have a turnover time of up to 120 days. Thus a very important role of ATII cells is to function as precursors of ATI cells.

The proteins podoplanin (also known as T1-alpha) and aquaporin (AQP-5) are present in the apical membrane and are commonly used markers for ATI (27), whereas pro- surfactant protein C (pro-SP-C) and surfactant protein A (SP-A) (29) are

markers for ATII cells. ATI cells are involved in modulation of immune functions, regulation of alveolar fluid volume, water fluxes and transcellular ion transport (27), functions that ATI inherit from their progenitor cells, ATII.

ATII are spherical pneumocytes of about 9 μm in diameter. They occupy only 4% of the peripheral epithelium, but constitute 60% of the cell number. An important homeostatic function of ATII cells is the synthesis and secretion of surfactant. In 1954, Macklin was the first to argue that ATII secreted surfactant from stores in lamellar bodies. Surfactant consists of mostly lipids (91%) of which 81% are phospholipids and 10% neutral lipids. The other part consists of 7% protein and 2% carbohydrates. Surfactant decreases the surface tension at the air liquid interface in the peripheral airways, which in turn decreases work required to inflate the lung, stabilizing the alveoli and preventing edema (30). Disaturated phosphatidylcholine is responsible for the surface tension properties of surfactant. Foreign substances such as drugs and environmental pollutants (xenobiotics) are metabolized by ATII cells. Metabolism of xenobiotics results in the formation of water soluble compounds which can then be cleared. In ATII cytochrome P-450-dependent monooxygenase system catalyses the monoxygenation of a variety of lipophilic substances in the presence of oxygen and NADPH (31). Clara cells also exhibit p450-dependent monooxygenase activity. ATII cells are in close proximity to the circulation and it is thought that xenobiotic metabolic functions apply to soluble organic compounds in blood and not just inhaled substances.

ATII also play a role in transepithelial water movement. It is important for alveolar function that the surface be dry, because excessive fluid would increase the distance

between the alveolar air space and the pulmonary capillary blood, thus decreasing gas exchange. ATII cell can also release large amounts of water and sodium across the plasma membrane (32).

- ***Lung epithelial cells: Role in asthma***

The cartilaginous bronchi and the membranous bronchioles function to maintain the more distal alveoli free of pollutants and pathogens. Coughing, sneezing and bronchoconstriction are some of the mechanisms used to protect the lungs. Secondly, at the air cell interface, the mucous acts as a filter. It allows for mucociliary clearance and is home for epithelial cell secretion products such as lactoferrin, secretory IgA and lysozyme. These mechanisms protect the respiratory units, allowing gas exchange. Surfactant also plays a role in the response to infections, enhancing bactericidal phagocytic activities of alveolar macrophages (33).

There is increasing evidence to suggest that alveolar epithelial cells play important roles in inflammatory reactions. ATII cells can induce, amplify and modulate inflammatory reactions by expressing several cytokines and chemokines and other mediators like IL-6, CXCL8 (IL-8), TNF (Fig. 1.3). Additionally, ATII cell can produce COX-2 (34), iNOS (35, 36) and MMP-9 (37). These mediators are involved in activation and recruitment of neutrophils and other cells to the site of inflammation. IL-6 is released early in inflammatory responses and augments antibody production. IL-8 has potent neutrophil recruitment and activating properties. TNF produced by ATII can stimulate further production of IL-8 and induce the expression of ICAM1, essential for neutrophil recruitment. Human primary ATI and ATII cells produce "monocyte chemoattractant protein 1" CCL2 (MCP-1) and "regulated on activation

normal T cell expressed and secreted" CCL5 (RANTES) (29). Both chemokines are upregulated by TNF and IFN-γ (38, 39). CCL2 is a chemoattractant for T cells, B cells and NK cells. Primary ATII cells also secrete growth-related oncogene-alpha CXCL1 (GRO-α) a potent chemoattractant for neutrophils. Interestingly A549 cells, an ATII cell line, also release CCL2 in response to TNF (40).

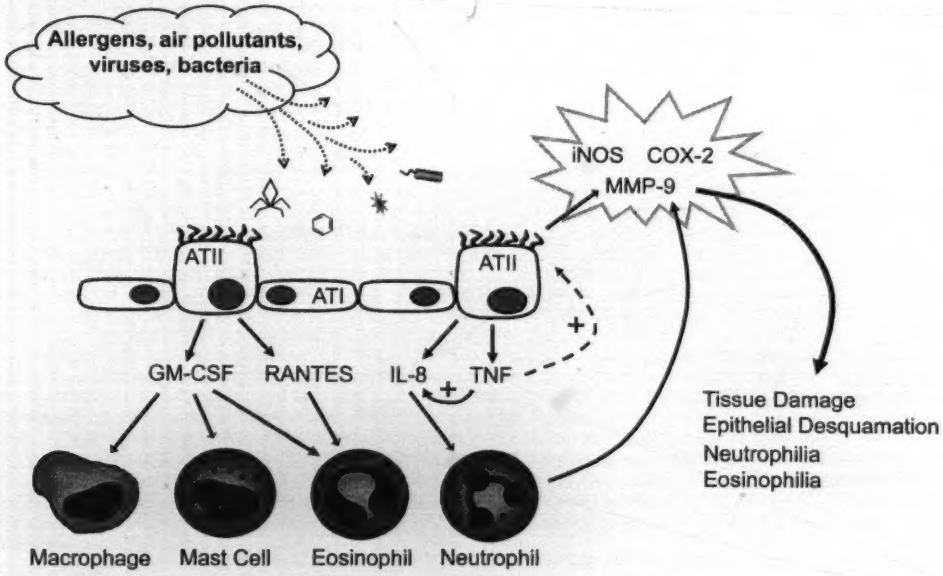


Figure 1.3: Role of alveolar type II (ATII) cells in inflammation. Airborne pathogens and pollutants can be inhaled and induce ATII cells to release proinflammatory mediators such as granulocyte-macrophage colony stimulating-factor (GM-CSF), regulated on activation normal T cell expressed and secreted (RANTES/CCL5), interleukin-8 (IL-8/CXCL8), tumor necrosis factor (TNF), COX-2, iNOS and MMP-9 all of which may cause or promote tissue damage.

Many proteases released by ATII cells interact in a regulatory manner to control other pro-inflammatory mediators. ATII also express cathepsin D which can specifically

cleave and inactivate CCL3 (MIP-1α) and CCL4 (MIP-1β) (41). MMPs 1, 3, 9, 13 and 14 inactivate CXCL12 (stromal cell-derived factor-1/SDF1) (42). MMP-9 also potentiates CXCL8 activity, degrades and inactivates CXCL1 and does not affect CCL5 or CCL8 (monocyte chemotactic protein-2/MCP-2) (43).

ATII derived MMP-9 can be substantial given the high number of ATII cells in the lungs, although there are other sources of MMP-9 in the lungs. Although the precise role of MMP-9 in ATII cells has not been established in humans, a study using knockout mice in a model of fibrosing alveolitis demonstrated that ATII cells are not regenerated after intratracheal bleomycin treatment in MMP-9 deficient mice (44). Authors suggested a model in which Clara cell migration into the site of alveolar injury is impaired in the absence of MMP-9. Besides the likely protective roles of MMP-9, overexpression of this enzyme can contribute to tissue remodeling, airway hyper responsiveness, leukocyte recruitment and epithelial desquamation as will be discussed in a later section.

Production of NO is a very important role of LEC in asthma. Epithelium-derived NO controls airway hyperresponsiveness (AHR), the main feature of asthma defined as an increase in the left shift of dose response to histamine or methacholine (45). Additionally, epithelium-derived NO controls ciliary beat frequency, submucosal gland secretion and ion channel activity (46).

- ***Nitric oxide: Role in asthma***

NO plays physiological and pathological roles in the airways. Low NO concentrations usually account for physiological roles which include bronchodilation and blood flow regulation, whereas at higher concentrations NO participates in cytotoxic and

cytostatic protective roles against pathogens (47, 48) and tumors (49). Interestingly, NO is detected in exhaled air in asthmatic patients, intriguing researchers as to what the role of NO is in this condition.

Macrophages, neutrophils, vascular endothelial cells and LEC are likely sources of NO in asthmatic patients. Upon stimulation with pro-inflammatory mediators such as TNF and IFN-γ these cells express iNOS, the enzyme responsible for elevated levels of NO production in asthma. Because NO is produced from multiple cell types in the lungs and because it can permeate plasma membranes reacting with a plethora of molecules and compounds, it is difficult to pinpoint the role of NO in asthma. Accumulated evidence indicates that NO might play a protective effect in asthma.

There are three physiological pathways by which NO can induce bronchodilation. Impaired relaxation of the airways in allergic inflammation might result from damage to these pathways, leading to the exacerbation of asthma. First, the excitatory and inhibitory non-adrenergic non-cholinergic neural system (eNANC and iNANC) mediate the bronchomotor tone in the airways (50). In humans, the iNANC relaxant response is mediated by NO (51) and this pathway is impaired in asthma (52). Secondly, it has been shown that bronchoconstriction induced by methacholine is reduced in guinea pigs treated with inhaled NO (53). This effect is also observed in asthmatic patients (54), thus providing a second pathway for NO-dependent bronchodilation. Thirdly, NO can act via sGC-independent pathways through the formation of nitrosothiols. Asthmatic patients have low nitrosothiol concentrations in the airways (55) and since these compounds are potent bronchodilators (56, 57) it is likely that low levels might influence asthma.

NO is also involved in the regulation of AHR. AHR was reduced by endogenous NO and potentiated in the presence of NOS inhibitors in guinea pigs (58). The relaxing effects of bradykinin, endothelin-1, substance P, and adenosine in intraluminal treatments of tracheae are NOS-dependent (59-63). Although these pathways are less understood in humans, it has been reported that NOS inhibitors in bradykinin-induced asthma potentiated the methacholine-induced AHR (64).

Interestingly, NO enhances methacholine and bradykinin-induced mucus glycoprotein secretions in isolated glands (65). Moreover, TNF-induced release of mucin in tracheal epithelial cells was inhibited in the presence of NOS inhibitors (66). Another function of LEC, ciliary movement, is NO-stimulated. Ciliary beat frequency is stimulated by TNF (67), through a mechanism that appears to involve iNOS; ciliary beating slows down in the presence of NOS inhibitors and restored by addition of L-arginine (46, 67). Finally, LEC are responsible for mucus liquid volume homeostasis. A number of ion channels regulate electrolyte transport and liquid volume in mucous. NO regulates at least two of these channels (apical anion and basolateral potassium channels) through a cGMP-dependent pathway (68) involving NO in the epithelium-mediated regulation of liquid volume.

The human lung epithelium, in response to pro-inflammatory stimuli such as TNF can generate NO. Another major mediator released by LEC in asthma is MMP-9. In the next section I will describe the roles of MMP-9 in the pathogenesis of asthma as an example of an inflammatory condition of the airways where MMP-9 plays a central role.

- ***Matrix metalloproteinase-9: Role in asthma***

The roles of MMPs in the pathogenesis of lung disease such as asthma and COPD have been studied extensively. MMP-9 is the predominant MMP in the airways of asthmatic patients (69). Other MMPs have been detected such as MMP-2 and -13 but they are present at significantly lower levels than MMP-9 (70, 71). In the normal lung MMP-9 levels are low or undetectable, however following various stimuli resident cells such as bronchial epithelial cells (72), Clara cells (73), ATII cells (37, 74, 75), fibroblasts (76), smooth muscle (77) and endothelial cells (78) produce MMP-9. ATI and neuroendocrine and goblet cells have not been shown to produce MMP-9. Leukocytes are recruited to the lungs during inflammatory conditions and have also been shown to produce MMP-9. Macrophages (79), eosinophils (80), mast cells (81), and lymphocytes (82) all produce MMP-9.

Asthmatic patients have higher levels of MMP-9 than normal individuals in sputum, bronchoalveolar lavage fluid and cultures of alveolar macrophages (83-85) but not in peripheral granulocytes (85). In a study where normal controls were compared with moderate asthmatic patients it was reported that asthmatic patients had increased expression of MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) in epithelial cells (86). In another study it was found that MMP-9 and TIMP-1 were upregulated from epithelial lining fluids in patients suffering from status asthmaticus (71). Multiple other studies have also documented the increased levels of MMP-9 in asthmatic patients and the correlation to severity of the condition (70, 87, 88). Moreover, the position of the MMP-9 at chromosome 20q11.1-13.1 is associated with bronchial hyperresponsiveness (89). All these studies have reported strong

correlations between MMP-9 activity and expression in the lung and severity of the disorder.

More evidence for the role of MMP-9 in asthma comes from animal models where specific MMP inhibitors reduce airway inflammation induced by allergen, LPS (90) or toluene diisocyanate (91). Additionally, ovalbumin-sensitized and challenged MMP-9 knockout mice display significantly less airway inflammation than wild-type mice (92). Subsequent studies of MMP-9 deficiency demonstrated the role of MMP-9 in dendritic cell (92-94), eosinophil and Th2 lymphocyte recruitment (95). Collectively, data from MMP-9 deficient mice and asthma patients indicate that MMP-9 contributes to airway inflammation and airway hyperresponsiveness.

The proposed mechanisms through which MMP-9 exerts its deleterious effects include the enhancement of influx of inflammatory cells into the airway mucosa, and activation of profibrotic factors such as TGF-β1 (96). It has also been proposed that MMP-9 is responsible for detachment of airway epithelial cells, chemokine released from ECM stores, and degradation of the basement membrane to facilitate extravasation of leukocytes.

2. Cyclooxygenase-2

Although Cyclooxygenase-2 (COX-2) was investigated as a mediator in this thesis, its study did not constitute a significant part of the major findings in our research.

A. The cyclooxygenase family

Over 75 years ago, prostaglandins (PG) were discovered as muscle-contracting components of human semen (97). Thirty years later, PGs structures were

characterized with the identification of $PGF_{1\alpha}$ and PGE_1; followed by the characterization of the two enzymes, COX-1 (98) and COX-2 (99, 100) that catalyze the formation of PG. Later elucidation that COX was the target for non-steroidal anti-inflammatory drugs (NSAID) (101) made COX an important focus of research. More recently, the existence of an alternative splice variant of COX-1 (named COX-3 or COX-1b) has been elucidated (102). The functional role in humans of this alternative form of COX-1 is still controversial (103).

B. Structure of cyclooxygenase

COX catalyses the first committed step in the synthesis of prostanoids, (PG, prostacyclin and thromboxanes). These enzymes (also called PGH synthases) contain two separate active sites that catalyze sequential reactions. The first reaction is the double-oxygenation of arachidonic acid (AA) which results in PGG_2 formation (an unstable intermediate) and the peroxidase reaction that converts PGG_2 to PGH_2. PGH_2 is released from COX and is converted to the various PG or thromboxane by their respective synthases.

COX enzymes are homodimers of 70kDa peptides and each subunit contains three domains namely the epidermal growth factor domain, membrane binding domain and the catalytic domain. There is a heme group in each subunit that is required for enzyme activity. The heme group is important in the activation of COX by $ONOO^-$ (104, 105). Interestingly, PGG_2 can itself activate COX once produced (106, 107).

COX-1 is the constitutive isoform of this family and it is involved in physiological functions such as cytoprotection of the stomach and platelet aggregation. COX-2 is

inducible and its upregulation takes place during acute inflammatory stimulation, although in some tissues like kidney, COX-2 is constitutively expressed. The two forms of COX carry out identical reactions, but COX-1 and COX-2 are only 60% identical in their amino acid sequence and as a result there are subtle differences. The first one is that while both isoforms are activated by $ONOO^-$, COX-2 requires tenfold less $ONOO^-$ than COX-1. This difference could mean that COX-2 will be preferentially activated in conditions of high NO and $O_2^{\cdot-}$. Moreover, COX-2 can use AA derivatives that COX-1 cannot catalyze such as arachidonylethanolamide (108) and 2-arachidonylglycerol (109).

D. Cyclooxygenase-2: role in asthma and septic shock

Inflammatory and structural cells of the lungs (e.g. LEC) are responsible for release of numerous mediators involved in clinical and pathological events in asthma (110). Among the most studied arachidonic acid metabolites we find PG (D_2, $F_{2\alpha}$, I_2), thromboxane A_2 (TXA_2) and cysteinyl leukotrienes (C_4, D_4, E_4). PG and TXA_2 are produced in the lungs by COX-2 in response to endotoxin and cytokine stimuli (LPS and TNF) (111, 112). These mediators act through their receptors to control bronchoconstriction, airway secretion, chemotaxis and AHR (110).

The roles of COX-2 products have also been studied in VSM. Under physiological conditions vascular tone is controlled by a monolayer of endothelial cells which form a barrier between the vessels and circulating blood. Endothelium-derived autacoids such as Endothelin-1, norepinephrine, angiotensin 1, thromboxane, PGI_2, and NO control VSM contractility (see Chapter I, 1B; for a detailed role of these mediators). In the presence of LPS however, COX-2 is upregulated in human VSM resulting in

elevated expression of PG (113) such as PGE_2 and TXA_2 as described earlier. The role of COX-2 in health and disease has been summarized recently (114).

It is well accepted that arachidonic acid metabolites play important roles in conditions where platelet aggregation, vasoconstriction, relaxation, and inflammation take place. As discussed above in both asthma and septic shock these characteristics are observed and it is not surprising that COX-2 has been a major pharmacological target in the treatment of many inflammatory disorders.

3. Matrix metalloproteinase-9

A. The matrix metalloproteinase family

Matrix metalloproteinases (MMP) are a family of zinc-containing endopeptidases that belong to the metzincin superfamily.. This superfamily also contains the ADAM group of enzymes (a disintegrin and metalloproteinase) originally thought to be transmembrane proteins with metalloproteinase-like activity. Thus the MMP family consists of 22 species encoded in different genes with a high degree of homology among the various members. The common domains are the Zn-containing catalytic domain, the pro-peptide domain (keeps the enzymes in a pro-form) and a carboxy-terminal hemopexin-like domain (important in substrate recognition). The family is grouped according to substrate specificity and protein localization. Collagenases include MMP-1, MMP-8 and MMP13, capable of degrading interstitial collagen. Gelatinases include MMP-9 and MMP-2 which are specific for denatured collagen (gelatin) and collagen-IV. Stromelysins such as MMP-3, MMP-10 and MMP-11 degrade non-collagen components such as fibronectin, laminin and vitronectin.

Membrane-type MMP (MT-MMP) are present on cell surfaces (MMP-14, MMP-15, MMP-16, MMP-17 and MMP-24). Other MMP are matrilysins (MMP-7 and –MMP-26), metalloelastase (MMP-12) and enamelysin (MMP-20). Unlike MMPs, ADAM contain a disintegrin-like domain, similar to disintegrins present in snake venom (115). Although there is a MMP-28; MMP-4, -5, -6, -18, -22 and -27 are not official MMP.

i. Regulation of matrix metalloproteinases

MMP are regulated at multiple levels including transcription (with the exception of MMP-2, which is produced constitutively (116), zymogen activation and inactivation through specific inhibitors called tissue inhibitor of metalloproteinases (TIMP). Gene expression is induced by cytokines, growth factors and integrin-mediated signalling (69). MMP are synthesized and secreted to later undergo enzymatic cleavage of their pro-domain to expose the catalytic domain. The pro-domain contains a cysteine that interacts directly with the zinc ion in the catalytic domain and proteolytic cleavage of the pro domain is required. Among the proteases that can activate MMP are elastase, other MMP, trypsin, plasmin and plasminogen activators. MMP are also regulated by TIMP. TIMP are a family of four polypeptides (TIMP-1 to -4) that bind to MMP and specifically inhibit their activity. They bind non-covalently in a 1:1 ratio. In the case of TIMP-1, -2 and -4 these peptides are found in soluble form, whereas TIMP-3 is bound to the extracellular matrix. Other proteins such as α2-macroglobulin can also interact with MMP and inhibit their activity in a non-specific way by preventing MMP from binding to their substrates (117).

C. Matrix metalloproteinase-9 structure and regulation

i. Gene and protein structure

The MMP-9 (gelatinase B) gene is on chromosome 20q11.1-13.1. The gene was cloned in 1989 from human lung fibroblasts (118) and later found to be equivalent to the type IV collagenase of neutrophils (119).

MMP-9 is synthesized as a proenzyme and it is the largest MMP at 92 kDa. In addition to common MMP domains, MMP-9 contains a fibronectin type II-like repeat within the catalytic domain which results in higher binding affinity for gelatin and elastin (120). Additionally, MMP-9 contains a type V collagen-like domain which is highly glycosylated, potentially conferring resistance to degradation and substrate specificity.

ii. Regulation

- ***Proteolytic activation***

MMP-9 like other MMPs requires proteolytic cleavage of the pro-domain to expose the catalytic domain. Removal of the pro-domain disrupts the interactions of a pro-domain cysteine which interacts directly with the Zn metal found in the catalytic pocket. This method of activation is known as the cysteine switch activation (121). In the case of MMP-9, a number of other MMPs can perform the catalytic cysteine switch activation (Fig. 1.4).

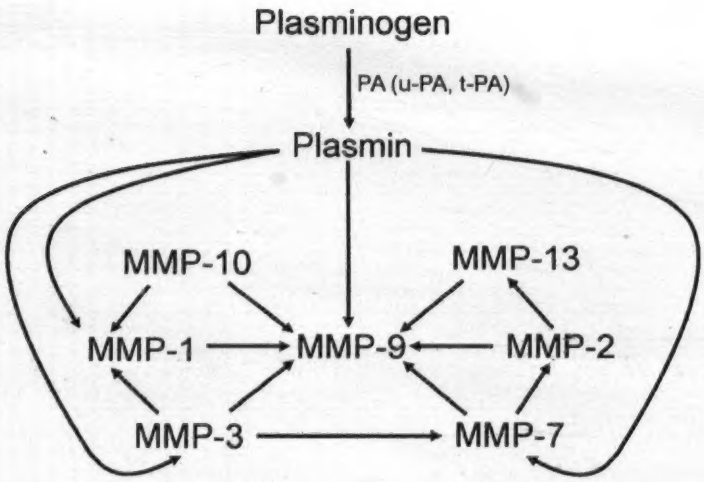


Figure 1.4: Plasmin and MMP involved in the proteolytic activation of MMP-9.

MMPs are differentially expressed according to the tissue of origin thus which MMP activates MMP-9 depends on the tissue or cell type in question. Because VSM and LEC express MMP-2 constitutively, and can also express MMP-3, and MMP-13, these are the most likely regulators of MMP-9 in these cells.

- ***Transcriptional regulation of MMP-9***

Although MMP-9 is regulated at multiple levels as described above, its transcriptional regulation is regarded as the most important mechanism dictating MMP-9 availability. Most of the literature regarding MMP-9 regulation is concerned with gene expression, however it is well documented that in the case of neutrophils, MMP-9 is stored at the end of neutrophil differentiation and secreted upon neutrophil activation from pre-stored tertiary granules. The MMP-9 promoter displays over 200 putative transcription factor sites within 1000 nucleotides of the 5' flanking promoter region when evaluated with the Transcription Element Search System software. But which

of these elements are functional cis-elements in the transcriptional regulation of MMP-9? In the next part we review the literature and summarize the reported functional components of the MMP-9 promoter/enhancer elements of MMP-9.

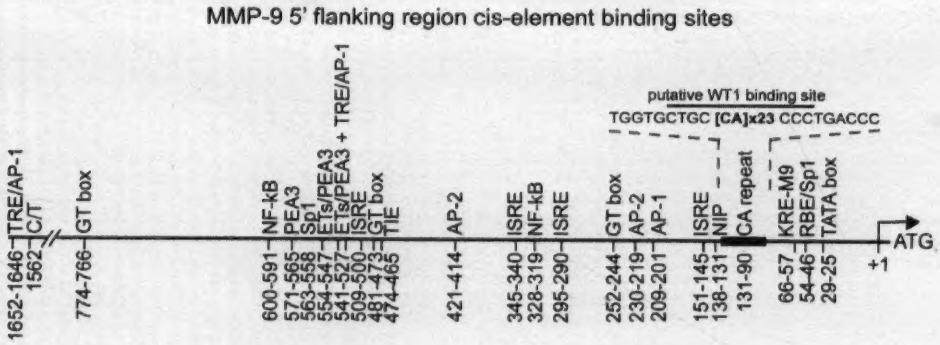


Figure 1.5: Functional transcription factor binding sites in the MMP-9 promoter. The line represents a 1650 nucleotide long region of the MMP-9 promoter. Above the line, the abbreviated names of the transcription factors are shown with the vertical numbers indicating the binding site relative to the ATG initiation site (+1). The black box indicates the CA repeat region, a putative WT1 binding site.

As shown in the Fig. 1.5, there are a number of characterized binding sites with functional relevance. The TATA box is at position -29. This site is essential for transcription initiation and is found in other MMP genes like MMP-1 and MMP-3, but absent in MMP-2. The nuclear stimulating protein 1 (Sp-1), also called GC box is present at two sites in position -562 and -54. At position -54 there is also a retinoblastoma binding element (RBE) or GT box. A TGF-β1 inhibitory element (TIE) is present at position -474. There are 3 different activator protein-1 (AP-1)

binding sites (also called 12-o-tetradecanoyl-phorbol-13-acetate (TPA)-responsive element (TRE)). AP-1 is the site for c-Fos and c-Jun binding (Fig. 1.6). The polyomavirus enhancer A-binding protein-3 (PEA3) is recognised by the ETS factor families which are activated through the MAPK pathways and downstream of PKC (Fig. 1.6). There are two NF-kB binding sites at positions -600 and -328 of the p65 and p50 subtypes respectively. A microsatellite element of CA repeats is found in position -131.

Upon stimulation with Ca^{2+} the keratinocyte element-4 relative-M9 (KRE-M9) and the nearby AP-1 are required for MMP-9 activation (122). The AP-1 elements are also important in phorbol ester and TNF induction of MMP-9, collaborating with the NF-kB and Sp1 sites (123). IL-1β acts through the activation of NF-kB and AP-1 (124). TNF-induced upregulation of MMP-9 is increased by an enhancer element from -600 to -500. In general the only elements that are considered essential for MMP-9 expression are the AP-1 sites at -79, NF-kB and Sp-1 sites (125). The pathways that transduce these signals are summarized in Fig. 1.6.

In addition to regulatory elements, there are two polymorphism sites in the MMP-9 promoter namely, the nucleotide substitution observed at position -1562 and the CA repetitive element found at position -131 (Fig. 1.5).

Replacement of the C by a T at position -1562 results in higher promoter activity (126). The CA repetitive element polymorphism is thought to alter DNA structure and affect transcription as it is close to the TATA box and other important regulatory sequences. The length of the CA repeat can vary between 14 and 27 repeats. Interestingly the most common lengths are 14, 21, 22 and 23 with lower tolerance for

16, 17, 18, 19, 20, and 24-27. The functional role of the CA repeat is inconclusive. However, in a study of African-American women, the CA_{14} repeat has been associated with increased risk of premature rupture of fetal membranes, thus potentially indicating that a repressor of the MMP-9 gene binds preferentially to longer CA repeats. Interestingly, it has been reported that a transcription factor called WT1 can bind to the CA repeat (see below).

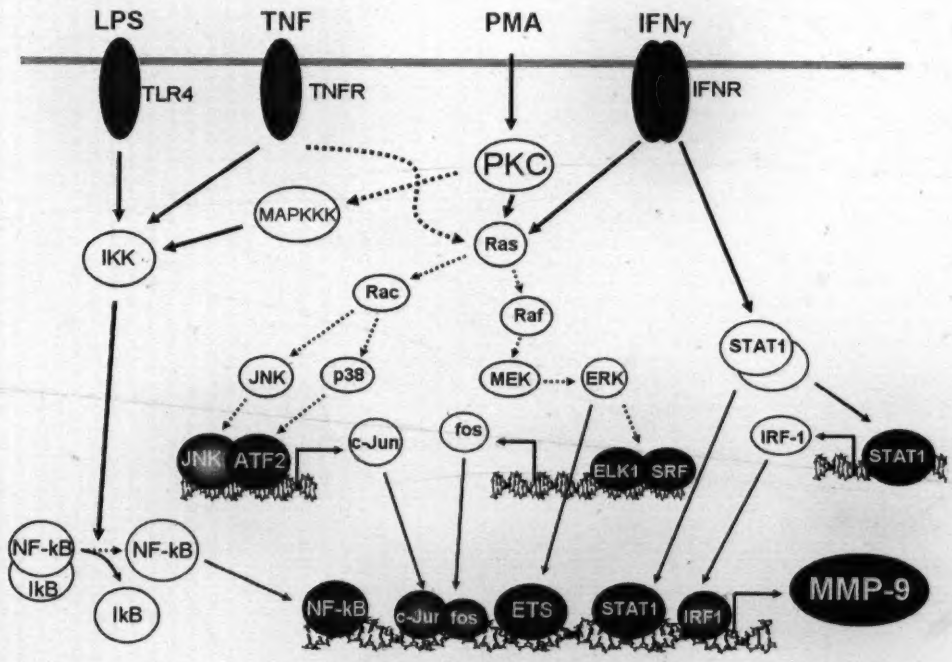


Figure 1.6: Summary of regulatory pathways involved in MMP-9 gene expression. Lipopolysaccharide (LPS) activates the toll-like receptor-4 (TLR4) resulting in the activation of NF-kB and its subsequent translocation to the nucleus. TNF binds to its receptor (TNFR), leading to the activation of NF-kB and mitogen activated protein kinase (MAPK) pathways through Ras. Activation of Ras optimally results in the activation and nuclear translocation of c-jun, fos and ERK, which bind

to the MMP-9 promoter at positions AP-1 and PEA3. Similarly, phorbol myristate acetate (PMA) also activates the Ras pathway through protein kinase C (PKC). Finally, IFN-γ activates its receptor (IFNR) which in turn activates STAT1. STAT1 binds to the MMP-9 promoter and also induces the expression of interferon regulatory factor-1 (IRF-1), also an important activator of the MMP-9 gene (125).

- ***Regulation by nitric oxide and peroxynitrite***

MMP-9 is regulated at multiple levels by NO; however these mechanisms result in opposing effects on MMP-9 production and activity. While NO by itself does not activate gene expression of MMP-9, LPS- or cytokine-induced MMP-9 expression is modulated by NO. This regulation takes place at three different levels including oxidation of the cysteine residue of MMP-9 pro-domain involved in the cysteine switch increasing activation (positive effect), reduction of the MMP-9 mRNA half-life (negative effect) and inhibition of NF-kB activation (negative effect).

Pro-MMP-9 chemical activation is evident in conditions present in the technique zymography, where both forms of MMP-9 (active and pro) degrade gelatin. Additionally, other classical MMP activators such as organomercurials induce activation of pro-MMP-9 independent of proteolysis. Thus chemical conditions in which the pro-domain interactions with the catalytic site are disrupted result in the formation of an active form of proMMP-9. Interestingly, in conditions of high NO levels a similar activation of pro-MMP-9 can take place, where NO activates pro-MMP-9 indirectly through the formation of $ONOO^-$. This pathway is independent of proteolytic activation and involves oxidation of the cysteine residue that participates in the cysteine-switch. $ONOO^-$-dependent oxidation of pro-MMP-9 disrupts the

interactions of the pro-domain with the catalytic site facilitating pro-MMP-9 access to substrates (125) (Fig. 1.7).

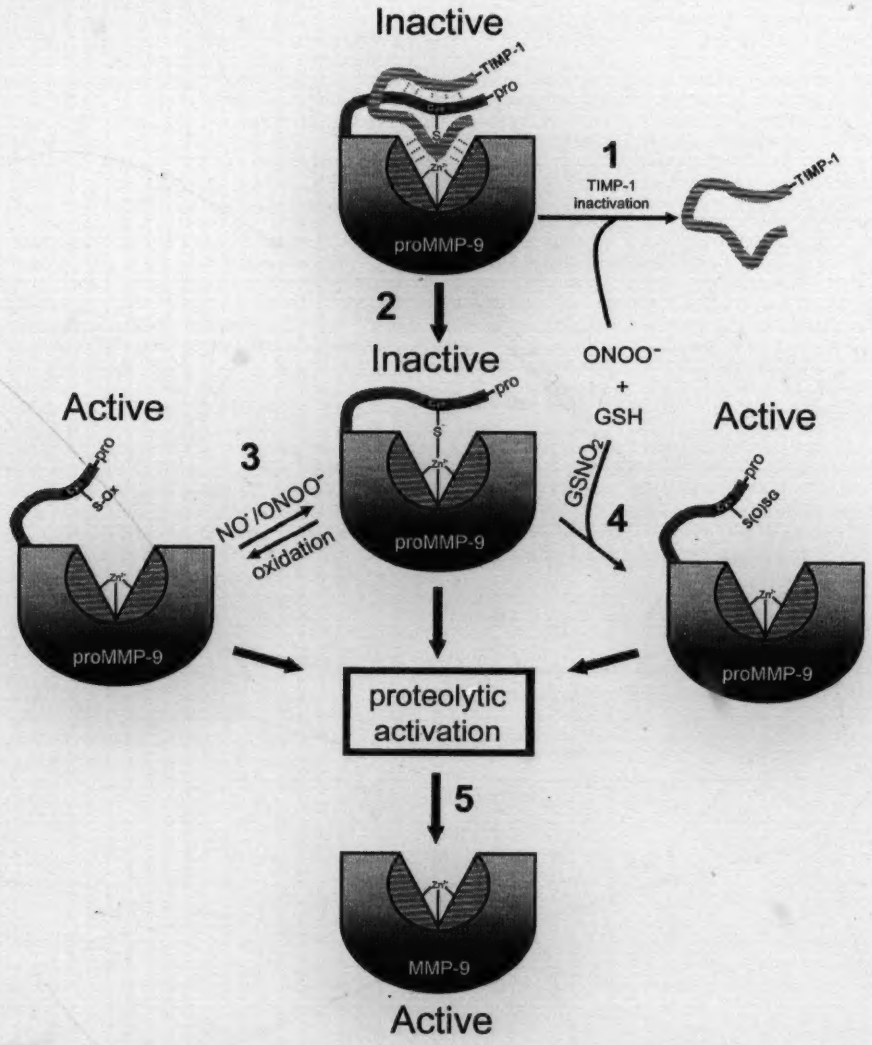


Figure 1.7: Mechanisms through which NO regulates MMP-9 enzyme activity. Inactive pro-MMP-9 is secreted in a complex with TIMP-1. TIMP-1 can be inactivated by $ONOO^-$ (1) to promote exposure of the pro-domain (2). NO may act directly through S-nitrosylation (3) of pro-MMP-9 or indirectly through the production of $ONOO^-$. The latter can activate pro-MMP-9 directly (3) or it can

interact with glutathione (GSH) to form glutathiolation-NO_2 ($GSNO_2$) (4). Pro-MMP-9 can also be proteolytically activated by other proteases (5).

In conditions of lower NO levels, S-nitrosylation of the pro-MMP-9 cysteine residue might also activate pro-MMP-9 (127) potentially in a reversible manner (Fig. 1.7). An alternative mechanism utilises the endogenous antioxidant glutathione (GSH), a tripeptide composed of the amino acids glutamate, cysteine and glycine. GSH contains a thiol group and since it is the most abundant thiol-containing factor in the respiratory tract (128), it plays an important role as a scavenger of $ONOO^-$ (129). However, it was reported that in conditions of persistent oxidative stress thiolation of the pro-domain cysteine residue requires ONOO- and GSH. $ONOO^-$ and GSH react to form a glutathione disulfide S-oxide [S(O)S] (Fig. 1.7) (130). In inflammatory lung diseases like asthma and acute respiratory distress syndrome, GSH levels decrease significantly to concentrations lower than 100 μM (131) as a result of higher concentrations of $ONOO^-$. Under these conditions pro-MMP-9 glutathiolation can take place. It has also been reported that $ONOO^-$ can inactivate TIMP-1 in a process that does not change the molecular weight of TIMP-1 (132) further substantiating the role of NO and $ONOO^-$ as promoters of MMP-9 activity. Interestingly NO can also inhibit NF-kB signalling, a process which might be part of a negative feedback loop to regulate the expression of iNOS (133).

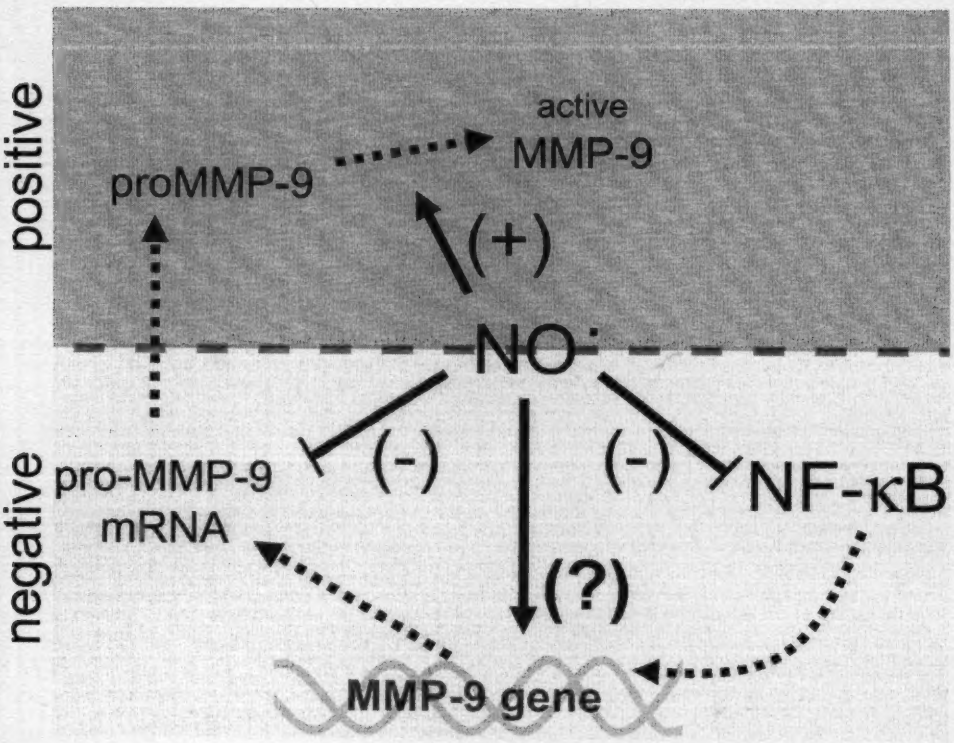


Figure 1.8: Dual role of NO in MMP-9 regulation. NO can chemically activate pro-MMP-9. However it can also reduce MMP-9 expression by interfering with MMP-9 mRNA stability and inhibiting NF-kB translocation to the nucleus. There are no reports of any direct effect of NO on MMP-9 gene expression (see question mark).

In contrast to the activating effects of NO described above, NO also decreases MMP-9 expression by reducing MMP-9 mRNA half-life (134). In addition NO also interferes with the NF-kB pathway, potentially inhibiting translocation of p50, thus preventing transcriptional initiation of MMP-9 (131) (Fig. 1.8). Although there are no reports on direct transcriptional roles of NO for MMP-9, it has been shown that MMP-1 expression in human melanoma cell lines is activated by NO through a MAPK-dependent pathway (135). It is thus likely that in VSM and LEC NO might

regulate MMP-9 gene expression either directly by modulating the MAPK pathway, or indirectly by interacting with MMP-9 mRNA, NF-kB, and pro-MMP-9 (Fig. 1.8). Because NO is an important regulator of MMP-9 it is likely that in inflammatory conditions such as septic shock and asthma NO regulates MMP-9 in VSM and LEC. As described earlier, these tissues express iNOS and MMP-9 and it is likely that iNOS-derived NO regulates MMP-9 in these tissues. The major source of NO during inflammation in VSM and human LEC is through its catalytic formation by iNOS. In the next part the NOS family of enzymes is discussed with an emphasis on iNOS and its regulation.

iii. Posttranscriptional fate of MMP-9 in the lung

- ***Expression and activity***

During development, rabbit LEC express MMP-9 at a low level during the glandular and canalicular stages. MMP-9 expression increases during alveolization where MMP-9 is synthesized by ATII cells (136). MMP-9 preferentially degrades gelatin but it has been reported to degrade a number of other proteins including TNF and IL-1α, summarized elsewhere (137).

- ***Degradation of MMP-9***

Other proteins reported to bind and inhibit MMP-9 activity include thrombospondins (138) and tissue factor protease inhibitor-2 (TFPI-2) (139). The most relevant protein by which MMP-9 activity is regulated is TIMP-1. Although all TIMP molecules have affinity for MMP-9, TIMP-1 is secreted complexed with MMP-9 (140). Moreover,

TIMP-1 has a unique interaction with MMP-9 because in addition to its binding to the catalytic domain TIMP-1 also interacts with the carboxy-terminal domain (141). Once the pro-domain of MMP-9 is removed, the active enzyme can be trapped by α2-macroglobulin and this complex is later removed by scavenger receptors.

4. Inducible nitric oxide synthase

A. The nitric oxide synthase family

Much has been said and written about NO. It is synthesized from L-arginine (L-Arg) and plays numerous roles as a regulator of cell function and communication. Over the last 20 years NO has been investigated extensively and yet it is difficult to define the exact role of NO. This diatomic radical is involved in multiple tumoricidal and anti-pathogenic processes yet despite its obvious protective roles in the immune system, NO has been implicated in the pathogenesis of many conditions (142).

Synthesis of NO from L-Arg takes place through the oxidation of its terminal guanidino nitrogen. This reaction is catalyzed by the nitric oxide synthase family (NOS) of enzymes. There are three different NOS isoforms each located in a different chromosome (143). Two of these isoforms are constitutively expressed and their activity is regulated by Ca^{2+} levels. The two constitutive NOS (cNOS) are nNOS and eNOS (also called NOS I and NOS III), named after the cell type in which they were originally discovered (rat neurons and bovine endothelial cells). The third NOS was named inducible NOS (iNOS/NOSII) since its expression is induced by pro-inflammatory stimuli.

B. Inducible nitric oxide synthase structure and regulation

i. Protein structure

All three NOS isoforms are comprised of an N-terminal oxygenase domain and a C-terminal reductase domain. The NOS polypeptides contain a calmodulin (CaM) binding site localized between the two domains and the enzyme core also binds tetrahydrobiopterin (BH_4), heme and L-Arg. The C-terminal reductase domain binds to FMN, FAD and NADPH. To catalyze NO synthesis, flavins take electrons from NADPH and transfer them to the heme iron, allowing heme to bind oxygen (Fig. 1.9). In a complex series of steps a single L-Arg molecule is transformed to L-citrulline and in the process generates a molecule of NO. All three NOS are homodimers and require CaM to dimerize, explaining the Ca2+ dependence of cNOS. In the case of iNOS, dimerization and CaM binding occur during enzyme folding in an irreversible way, thus iNOS functions in a Ca^{2+}-independent way.

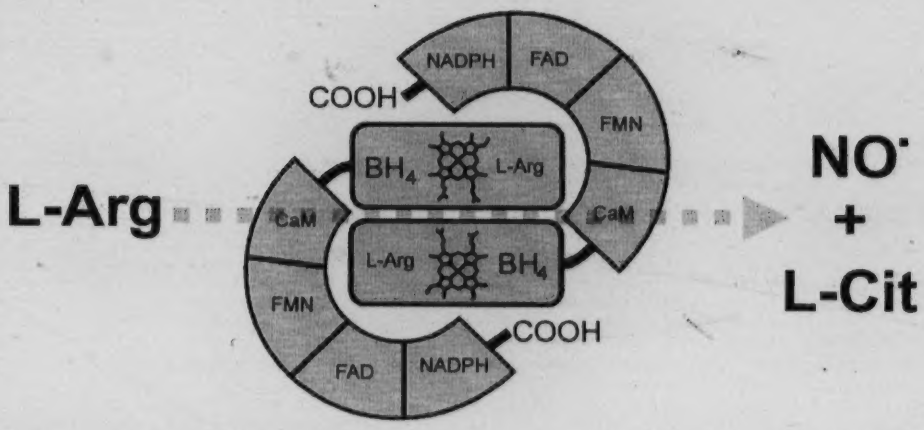


Figure 1.9: Domain structure and necessary cofactors of the nitric oxide synthase family. All three NOS isoforms form dimers. The reductase domain contains binding sites for nicotinamide-adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and calmodulin (CaM). The oxygenase domain binds to tetrahydrobiopterin (BH_4), heme and L-arginine (L-Arg). Every L-Arg molecule generates a molecule of NO and a molecule of L-citrulline (L-Cit). Oxygen is not shown.

ii. Regulation

NO is an unstable radical but is a small molecule that carries no charge. iNOS derived NO diffuses freely across cell membranes thus not requiring a transport system. A number of cell types can be induced to express iNOS. This enzyme was first isolated from murine macrophages in 1991 (144, 145). Contrary to nNOS and eNOS, iNOS does not require Ca^{2+} thus its function is regulated mainly at the transcriptional level. In untreated cells iNOS expression is undetectable or very low. Among the first pro-

inflammatory mediators that were used to stimulate iNOS expression, LPS, IL-1, IFN-γ and TNF were found to be potent iNOS gene activators. Besides LPS and cytokines, other factors that can stimulate upregulation of iNOS include forskolin (a cAMP-upregulating chemical), phorbol 12-myristate 13-acetate, PMA (protein kinase C stimulating agent) and growth factors such as platelet-derived growth factor (PDGF). Conversely cytokines like IL-4 (146), IL-8 (147) and IL-10 (148) are inhibitors of iNOS. Factors that inhibit the expression of iNOS include NF-kB inhibitors (diethyl dithiocarbamate and pyrphostin dithiocarbamate) (149, 150) and tyrosine kinase inhibitors (genistein, herbimycin A and tyrphostin) (151). Glucocorticoids are also inhibitors of iNOS induction (152).

C. Nitric oxide chemistry

Because of the reactive nature of the NO chemistry this free radical can undergo numerous reactions including those that generate reactive nitrogen oxide species (RNOS). Thus in an attempt to categorize the biological significance of these reactions, they have been divided into two groups of direct and indirect effects.

Direct effects involve the interactions of NO with iron-containing proteins. The most common reaction is between NO and heme-containing protein such as soluble guanylate cyclase (sGC). These reactions require low concentrations of NO and are thought to account for most biological effects of NO. In contrast indirect effects involve RNOS and can be further subdivided into oxidative and nitrosative stress depending on whether NO reacts with O_2 or O_2^-. Oxidative stress occurs from the reaction of NO with superoxide (O_2^-) and the formation of peroxynitrite ($ONOO^-$). Nitrosative stress involves the reaction with NO and oxygen (O_2) resulting in the

formation of dinitrogen trioxide (N_2O_3). Together indirect effects of NO can result in DNA and protein damage, inhibition of DNA repair and thiol group nitration. Thus it is thought that NO production at lower than 1 μM mediate direct effects, whereas higher concentrations for extended periods of time can result in indirect NO effects (153).

Among the most widely known direct effects of NO are its biological functions as a neurotransmitter and vasodilator (154, 155). These reactions are induced through the NO-dependent activation of soluble guanylate cyclase (sGC). NO binds to sGC heme iron activating the enzyme to catalyze the formation of cyclic guanosine 3',5'-monophosphate (cGMP) from guanosine 5'-triphosphate (GTP). There are three known targets for cGMP which mediate the signal transduction of the NO pathway downstream from sGC: cGMP-dependent protein kinase (PKG) (156), cGMP-regulated phosphodiesterases (157) and cGMP-gated ion channels (158). Additionally, cGMP may also activate PKA resulting in its translocation to the nucleus. PKA phosphorylates many transcription factors to mediate transcriptional regulation summarized elsewhere (159). This mechanism could mediate the NO-dependent regulation of gene expression. One of PKA reported targets is a transcription factor called WT1 and in the next section the relevant literature will be discussed.

5. Wilms tumor 1

A. Wilms tumor: An introduction

Wilms tumor (WT), originally described by Dr. Max Wilms and also called nephroblastoma is a pediatric kidney cancer that occurs in 1 in 10 000 children. It is

responsible for 8% of childhood cancers (160). The locus for the WT gene in chromosome 11 encodes a transcription factor (161-163) that was inactivated in WT (164). Thus this transcription factor was suspected to be a tumor suppressor gene involved in the pathogenesis of WT and named WT1, Wilms tumor 1 gene. However it was later found that only 10 to 15% of patients develop WT as a result of mutations in the WT1 gene, emphasizing the relevance of other genes in the pathogenesis of WT (165-167). Interestingly, other syndromes involve mutations in WT1 including, WAGR (WT, aniridia, genitourinary, mental retardation), Beckwith-Wiedemann, Denus-Drash and Frasier syndromes (168). Although the role of WT1 in the pathogenesis of these disorders is not fully understood, WT1 is involved in numerous cell processes including differentiation, proliferation and apoptosis (164). Up to 24 WT1 isoforms can potentially be produced but unfortunately little is known about the properties of WT1 variants.

B. Wilms tumor 1 structure and regulation

i. Gene and protein structure

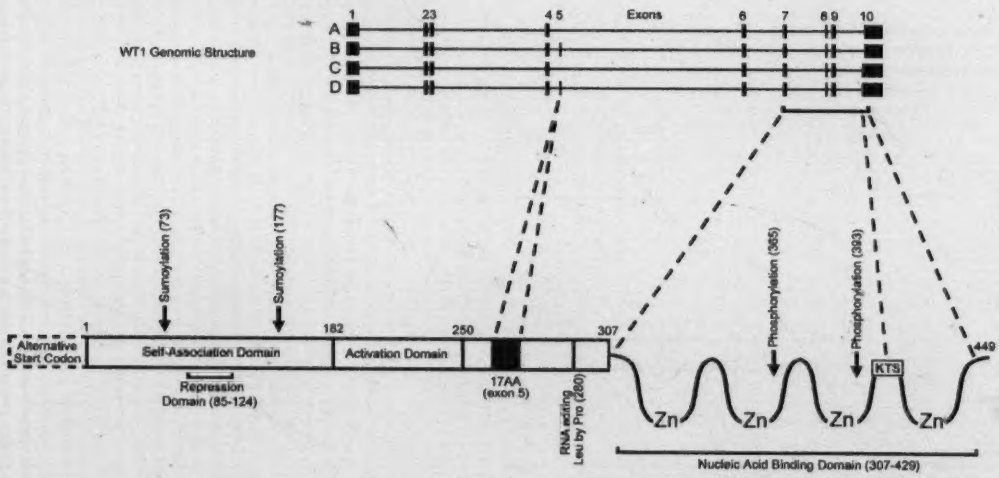


Figure 1.10: Wilms tumor 1 (WT1) gene structure and protein domains. The WT1 contains 10 exons that code 4 WT1 isoform variants (A, B, C and D). They differ in the presence/absence of exon 5 (17 amino acids) and a 3 amino acid insert (KTS) between Zn-fingers 3 and 4. All WT1 variants contain the repression domain, activation domain and 4 Zn-fingers.

Haber *et al* were the first to describe alternative isoforms of WT1 (169). Additional WT1 isoforms have been identified over the last 20 years. These isoforms are all encoded from a single 10 exon gene at position 11p13 (Fig. 1.10). The gene generates four isoforms A, B, C and D. The main structural differences among these four isoforms are the presence or absence of exon 5 (see below) and the insertion of three amino acids between zinc fingers 3 and 4. Thus isoforms A and C lack exon 5 and

isoforms A and B lack the three amino acid insertion (Fig. 1.10). WT1 isoforms use the same reading frame and display similar domains such as the nucleic acid binding domain, activation domain, repression domain and self-association domain. The first nomenclature used for WT1 isoforms (e.g. WT1A-D) was replaced by a more descriptive terminology as more WT1 isoforms were identified. However GreenBack continues to list WT1 isoforms as WT1A, B, C and D.

Each zinc finger is encoded independently by exons 7 to 10 (Cys_2-His_2 type zinc fingers). Between exons 9 and 10 there is an alternative splice donor site that inserts three amino acids, lysine, threonine and serine (KTS) giving rise to two different WT1 isoforms named WT1 -KTS and WT1 +KTS –T7-93- (Fig. 1.10). Exon 5 can also be alternatively spliced resulting in the exclusion of 17 amino acids. Thus WT1 may contain isoforms lacking both inserts known as WT1 (-/-) of 52 kDa or displaying both inserts WT1 (+/+) of 54 kDa as well as the combinations of lacking exon 5 but containing the KTS called WT1 (-/+) or the opposite WT1 (+/-).

The function of exon 5 (17 amino acids) is incompletely known. On one hand it appears to contain a repressor sequence, but others have found that it can work as a transactivation domain through factors such as prostate apoptosis response gene-4 (Par-4) (170).

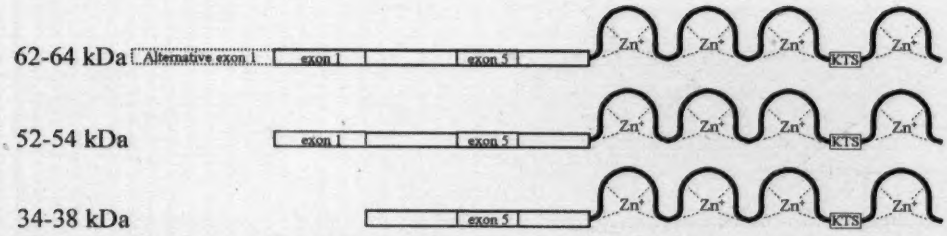


Figure 1.11: WT1 major molecular weight (MW) alternative isoforms. (Top) WT1 largest MW form contains an alternative exon 1 resulting in a 62-64 kDa protein. (Middle) The 52-54 kDa WT1 isoform. (Bottom) Smaller MW isoform lacks exon 1 and is 34-38 kDa. All the above forms can have or lack exon 5 and the KTS insert.

These isoforms use a common AUG initiation codon. However, there are two alternative initiation sites. The first one is a CUG which results in WT1 isoforms of 62-64 kDa (171). The second alternative initiation codon is an AUG within intron 1 at nucleotide 127 that generates WT1 isoforms of 34 to 38 kDa (172, 173). The 34-38 kDa WT1 forms lack the repression domain and may act as constitutively activated WT1 forms (Fig. 1.11).

WT1 can function either as a repressor or an activator. The repression domain has been localized to amino acids 85-124 and the activation domain to amino acids 182-250 (174) (Fig. 10). Since WT1 can interact with co-regulators, the underlying mechanisms of transcriptional regulation might be more complex (Fig. 1.12). There is a report that the 17 amino acid exon 5 product can also function as a repressor domain (175). Thus it is likely that different WT1 isoforms have different roles defined by the domains.

ii. Protein-protein interactions

Identification of WT1 target genes is difficult since WT1 recognises multiple DNA binding sites and can also bind to other transcription factors to function as a co-regulator (Fig. 1.12). WT1's effects on target genes are thus context and isoform dependent.

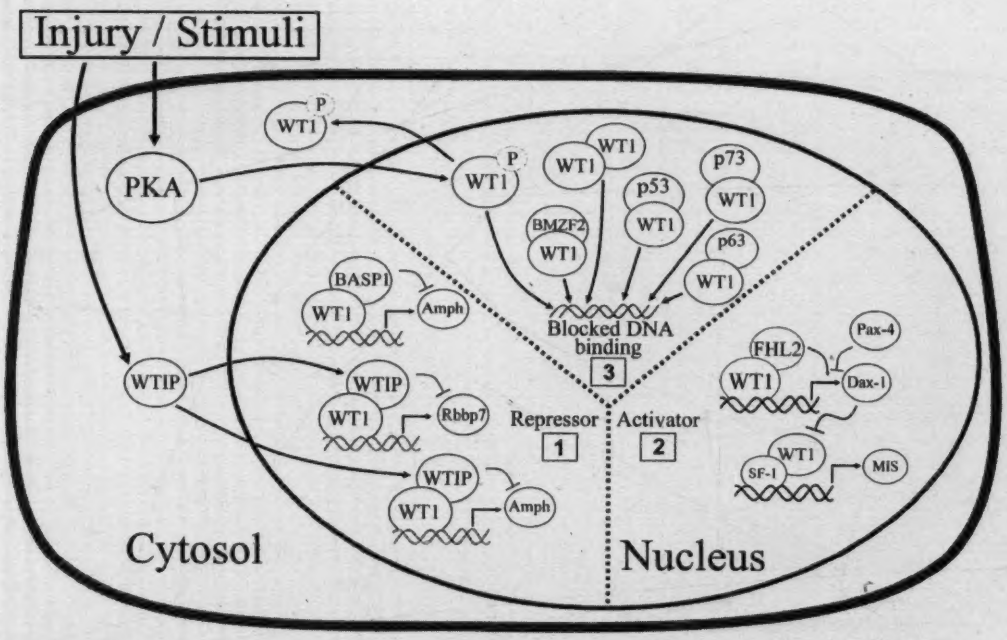


Figure 1.12: WT1 regulatory mechanisms. WT1 interactions are divided into three groups according to WT1's effect on the target gene and the mechanisms that regulate WT1 activity. WT1 can function as a repressor [1] through interactions with co-repressors brain acid soluble protein 1 (BASP1) and WT1-interacting protein (WTIP). Repression in this case has been demonstrated in the genes amphiregulin (Amph) and retinoblastoma binding protein (Rbbp7). Alternatively, WT1 can function as an activator [2] by either utilising Four and half LIM domain 2 protein (FHL2) as a co-activator or by acting as a co-activator itself as in the case of Mullerian inhibiting

substance (MIS) activation. Finally, WT1 DNA-binding might be impaired though dimerization events or by phosphorylation by PKA. In this case the effect on the target gene, either activation or repression, is reversed through the blockade of WT1 DNA-binding.

A number of transcriptional co-regulators have been reported to function as trans-activators/repressors. Brain acid-soluble protein 1 (**BASP1**) binds to the suppression domain of WT1 to function as a co-repressor of amphiregulin (176). Interestingly BASP1 is highly expressed in human adult lungs making it a potential co-repressor in ATII.

The WT1 interacting protein (**WTIP**) co-precipitates with WT1 from nuclear fractions and this interaction inhibits the activation of amphiregulin (177). In a podocyte injury model using puromycin aminonucleoside, WTIP shuttles from cell-cell interface to the nucleus to form a complex with WT1 (178). In this model, WTIP acted as a co-repressor of the retinoblastoma binding protein (Rbbp7). It would be interesting to study the expression of WTIP in ATII cells and its changes in micro-localization in LEC injury models.

A protein called bone marrow zinc finger 2 (**BMZF2**) is a large, putative transcription factor containing 18 Zn fingers that can bind to WT1 through Zn-fingers 6 -10 (179). WT1 binding to BMZF2 terminates WT1 repression functions.

WT1 has been co-immunoprecipitated with **p53** in WT patients and the interaction takes place through WT1 Zn finger regions (180). This interaction can enhance p53 binding to its DNA consensus sequence and inhibits p53-mediated apoptosis (181).

This interaction appears to transform WT1 from an activator to a repressor in a reporter gene construct (180).

Homologues of p53, namely **p73** and **p63** can also bind to the Zn finger region of WT1, inhibiting DNA binding by WT1, interfering with WT1's transcriptional roles.

WT1 can self dimerize or self-associate through its N-terminal domain (182-184). This interaction can occur between wild type WT1 and mutated allelic versions of WT1 creating heterodimers with a dominant negative effect.

Alternatively, instead of binding to DNA or to WT1 itself, WT1 can interact with DNA-binding transcription factors and function as a co-regulator. The steroidogenic factor 1 (**SF-1**) uses WT1 as a transcriptional co-activator to promote expression of Mullerian inhibiting substance (MIS), a hormone important in sexual development (185). This interaction is inhibited by direct competition of Dax-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on X-chromosome, gene 1) with WT1, which binds to SF-1 preventing WT1 association (185). Additionally WT1 can promote the regulation of Dax-1 acting through the co-activator four-and-half LIM-domain protein (FHL2) (186). In this molecular switch, WT1 can function as an activator and as a co-activator (Fig. 1.12).

Sterol-responsive element-binding proteins (**SREBP**) are a family of at least three transcription factors thought to play a critical role in lipid homeostasis by upregulating genes such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD-1) (187). WT1 binds to SREBP to co-repress those genes normally activated by SREBP (188).

WT1 can induce expression of heat shock protein 70 (Hsp70) by potential interaction with the transcription factor heat shock factor (HSF) in rat podocytes (189). In these cells Hsp70 associated with WT1 to form a complex but the authors could not determine the relevance of this interaction.

The prostate apoptosis response gene-4 **(Par-4)** interacts with the 17 amino acid region of exon 5. Par-4 has been implicated in neuronal development and pathogenesis together with **BASP-1** and can inhibit expression of dax-1 by binding to WT1 and acting as a repressor, in an opposing manner to that of FHL2 (Fig. 12) (190). Other proteins known to interact with WT1 have been reviewed elsewhere (190).

iii. Protein-RNA interactions

Due to its ability to bind RNA and associate with components of the splicing machinery, it has been speculated that WT1 may play a role in RNA processing. For example WT1 can bind to the IGF-II mRNA through its Zn finger domain (191). However, the role of such binding has not been studied. Using a random RNA pool in an effort to identify the natural mRNA ligands for WT1 it was shown that WT1 has affinity for different mRNA sequences (192).

WT1 colocalizes with interchromatin granules and splicing factor. Interestingly WT1 +KTS localized to nuclear speckles whereas WT1 –KTS has a diffuse nuclear distribution. In this study WT1 was co-immunoprecipitated with proteins of the spliceosome and co-purified with nuclear Poly(A)+ ribonucleoprotein (193). Additionally, WT1 associates directly to U2AF65 as part of the spliceosome (194). An RNA recognition motif is present in all WT1 isoforms (195).

iv. WT1 chemical modifications

Proteins can undergo a number of post-translational chemical modifications that may result in changes of activity, sub-cellular localization and degradation. Chemical modifications occur post-translationally thus allowing a fast response in a transcription-independent manner. These modifications include phosphorylation, nitration, nitrosylation, glycosylation, sumoylation and others.

Sumoylation is a modification thought to affect nuclear proteins resulting in repression of their transcription factor function. SUMO stands for small ubiquitin-related modifier that is involved in the covalent attachment to a target protein. This chemical modification is reversible though the action of isopeptidases that hydrolyze the isopeptide bond to remove the SUMO group. Sumoylation of WT1 occurs at two lysine residues in the N-terminus domain of WT1 (196). Although sumoylation of WT1 was not linked to any functional role, this modification can have several effects on transcription factors such as stimulation of DNA-binding activity (heat shock factors) (197) or down regulation of their transcriptional effects (c-Myb, c-Jun, p53) (198, 199).

WT1 can be phosphorylated by protein kinase A, a modification that results in loss of DNA-binding by WT1 and shuttling of WT1 from the nucleus to the cytosol (200-203). WT1 isoforms (+KTS and –KTS) are both translocated to the cytosol. Translocation is triggered by phosphorylation at Zn fingers 2 and 3 in Ser-365 and Ser-393 respectively. Point mutation of these sites results in retention of WT1 in the nucleus and WT1's binding to DNA. Ser-365 and Ser-393 mutated WT1 become unresponsive to PKA activity. Thus PKA is a critical regulator of WT1 function.

There are three potential tyrosine residues and eight potential cysteine residues that can be targets for nitration and nitrosylation respectively. However, there are no reports for WT1 nitration or nitrosylation.

v. WT1 DNA binding sites

Three different types of DNA binding site for WT1 have been described in humans, namely a GC-rich DNA-element, a TCC repeat and a CA repeat (175, 204, 205). The GC and the CA repetitive elements appear throughout the human genome and because until recently no transcription factors had been identified that recognise these repeats, they were thought of as "junk DNA" (206). CA repeats are the most abundant and polymorphic repeat element in the human genome and when the discovery was made that CA repeats of greater than 13-mer could specifically interact with nuclear factors (207) interest to characterize this dinucleotide repeat was created. For example, CA repeats are important in the regulation of insulin and Nramp genes (206).

A phylogenic analysis of 10 kb spanning the human MMP-9 gene revealed a highly conserved CA repetitive element reported to vary between 12 and 23-mers (125). Thus we hypothesized that WT1 could bind to this element and regulate MMP-9 gene expression.

vi. WT1 gene targets

With increasing understanding of the mechanisms mediating WT1 activity, and knowledge of DNA consensus sequences recognized by WT1 and transcription factors that use WT1 as co-regulators, the list of potential target genes influenced by WT1 has expanded dramatically. Binding site identification experiments with

oligonucleotides, whole genomic PCR and DNase I footprint analysis have also been used to search for potential WT1 targets. The current understanding of gene targets of WT1 ranges from growth factors and their receptors to other transcription factors. An extensive compilation of potential WT1 DNA binding sites has been summarized by Menke, A.L *et al* (208). A summary of some relevant sequences is shown in table. 1.1.

Table 1-1

DNA binding site	Technique	Reference
CGCCCCCGC	Oligonucleotides	(205)
CGGTT **[CA]x15** AATACACGCACAC	Whole genomic PCR	(209)
ACAT **[CA]x20** ACAC	Whole genomic PCR	(204)
[CA]x16 GTTAT	Whole genomic PCR	(204)
TTCTCCC **[CA]x9** GC **[CA]x10** A	Whole genomic PCR	(204)
CTGGAGCGGGGGCGGGG	DNaseI footprinting	(210)

Table 1.1: WT1 DNA binding sites from Menke A.L. *et al.* A number of different techniques have been used to determine these sequences. Four of these sequences contain CA dinucleotide repeats found in the MMP-9 promoter.

At least 4 DNA sequences containing CA repeats have been reported to bind WT1 (204, 209). However, there are no reports in the literature concerning WT1 involvement in the regulation of MMP-9.

vii. WT1 expression

In humans, WT1 expression in nuclei of podocytes has been seen as early as week 7 of gestation (211). In adults WT1 expression continues to be high in podocytes (211). WT1 is also expressed in embryonic and adult uterus, Sertoli cells and the epithelium and granulosa cells of the ovary. WT1 is also expressed in the spleen and the mesothelium, interestingly at a time when these tissues are switching from mesenchymal to epithelial, where WT1 may play a role in differentiation and proliferation in epithelium morphogenesis. WT1 is present in liver, thymus, brain and spinal cord. Other studies have shown WT1 expression in stem cells of the bone marrow, during development of the retina, and the proliferating coelomic epithelium and in the heart (208). It has been shown that WT1 is required for the formation of the olfactory epithelium in mice (212). Interestingly, a number of human epithelial cells express WT1. Adult epithelial cells including podocytes, epithelia cells of the ovaries (211), and breast epithelial cells (213) express WT1, suggesting a homeostatic role in these tissues. Although WT1 expression in adult human lungs has not been shown, several studies have demonstrated its expression in adult rat and mouse lung by Northern blot analysis (214-216), immunohistochemistry (217) and RNase protection assays (169).

6. Synthesis and conceptual model

In resting conditions, structural cells like VSM and LEC do not produce MMP-9. Stimulation of these tissues with LPS and cytokines results in the upregulation of iNOS and MMP-9 (Fig. 1.1 and 1.3). VSM and LEC cells express pro-inflammatory

mediators that contribute to tissue damage and interact to amplify inflammatory reactions. There is evidence indicating that iNOS and MMP-9 can cross-talk to modulate their inflammatory roles. Among the studied pathways for NO-dependent regulation of MMP-9, transcriptional regulation has not been investigated (Fig. 1.8). To study the potential interactions and possible cross talk between iNOS, COX-2 and MMP-9, we will use rat aortic VSM cells (A7r5) stimulated with LPS, IFN-γ, and phorbol 12-myristate13-acetate (PMA); individually or a combination. These stimuli should be sufficient to generate a septic shock model using VSM cells.

We anticipate that NO will be an important component in the LPS/cytokine-induced upregulation of MMP-9 and COX-2. Thus treatment of VSM cells with the iNOS inhibitors L-NAME and 1400W should have an inhibitory effect on the expression of COX-2 and MMP-9. However, it is possible that the MMP-9 mRNA levels could be reduced in the presence of iNOS inhibitors, since NO reduces MMP-9 mRNA half-life (Fig. 1.8). In conditions such as asthma and septic shock, high NO levels correlate with MMP-9 and COX-2 over-expression, thus it is possible that NO will have a positive effect on the expression of these genes.

These experiments will also be carried out in human LEC. Human LEC, in particular ATII cells, express iNOS, COX-2 and MMP-9 in inflammatory conditions like asthma (Fig. 1.3). The A549 ATII cell line will be subjected to experiments as described above to induce the expression of these three enzymes. We anticipate that similar findings will be observed. The rationale behind testing this hypothesis in A549 also involves the evaluation of our hypothesis in a human cell line since A7r5 is a rat cell line. Testing our hypothesis in a human cell will strengthen the value of our

findings. We anticipate that MMP-9 will be upregulated in the presence of TNF and that blockade of iNOS may result in a decrease of MMP-9 gene expression.

It has been demonstrated that NO can promote transcriptional upregulation of a variety of genes through the activation of PKG and PKA. Interestingly, the MMP-9 promoter contains putative binding sites for potential targets of PKA activation such as WT1 a gene repressor of Amph and Rbbp7 (Fig. 1.12).

Human LEC, like VSM, also contribute to inflammation by producing iNOS and MMP-9. In conditions like asthma, NO levels correlate with an increase production of MMP-9. It is reasonable to postulate that iNOS-derived NO can play a role in MMP-9 transcriptional activation. The mechanisms that mediate this pathway can involve sGC downstream targets like PKA and PKG. The PKA-regulated transcriptional repressor WT1 is likely to control MMP-9 gene expression downstream of NO.

Finally, WT1 expression will be studied in leukocytes, in particular lymphocytes. Lymphocyte proliferation is regulated by macrophage-derived NO and I will test whether WT1 levels or subcellular localization change in correlation with NO-dependent inhibition of lymphocyte proliferation.

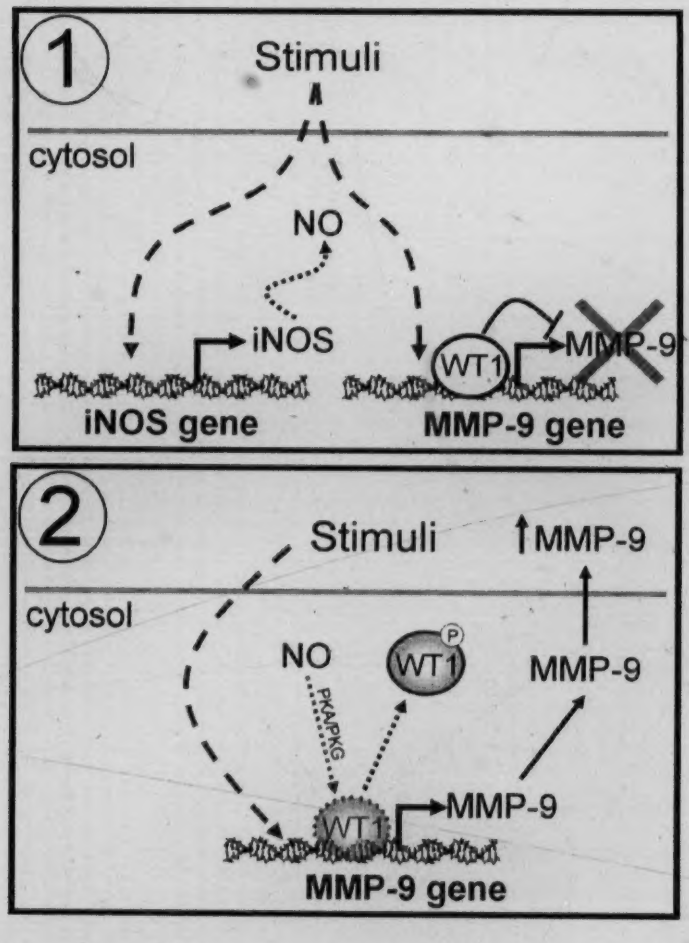


Figure 1.13: Conceptual Model.

In this conceptual model (Fig. 1.13), I hypothesize that upon stimulation, cells (VSM/LEC) are activated to express iNOS and MMP-9 and other pro-inflammatory enzymes like COX-2. Initially, MMP-9 is under the repression of WT1 and the stimulus is insufficient to induce MMP-9 transcription. NO levels activate sGC with subsequent activation of PKG and PKA. These kinases phosphorylate WT1, resulting in WT1 translocation to the cytosol and derepression of the MMP-9 gene. At this point the de-repressed MMP-9 gene is activated by the appropriate stimuli.

7. Hypothesis

iNOS-derived NO upregulates the transcription of COX-2 and MMP-9.

8. Objectives

1. To establish a septic shock model in Rat VSM by inducing co-expression of COX-2, iNOS and MMP-9.

2. To study the role of iNOS-derived NO in the transcriptional regulation of COX-2 and MMP-9 in rat VSM.

3. To determine if the interactions apply to human LEC.

4. To characterize WT1 expression in human LEC.

5. To determine the mechanisms by which WT1 regulates MMP-9 promoter.

6. To study WT1 expression in leukocytes.

8. References:

1. Kilbourn, R.G., D.L. Traber, and C. Szabo. 1997. Nitric oxide and shock. *Dis Mon* 43:277-348.

2. Szabo, C., C. Thiemermann, C.C. Wu, M. Perretti, and J.R. Vane. 1994. Attenuation of the induction of nitric oxide synthase by endogenous glucocorticoids accounts for endotoxin tolerance in vivo. *Proc Natl Acad Sci U S A* 91:271-275.

3. De Kimpe, S.J., M. Kengatharan, C. Thiemermann, and J.R. Vane. 1995. The cell wall components peptidoglycan and lipoteichoic acid from Staphylococcus aureus act in synergy to cause shock and multiple organ failure. *Proc Natl Acad Sci U S A* 92:10359-10363.

4. Kengatharan, M., S.J. De Kimpe, and C. Thiemermann. 1996. Analysis of the signal transduction in the induction of nitric oxide synthase by lipoteichoic acid in macrophages. *Br J Pharmacol* 117:1163-1170.

5. Bucher, M., K.P. Ittner, M. Zimmermann, K. Wolf, J. Hobbhahn, and A. Kurtz. 1997. Nitric oxide synthase isoform III gene expression in rat liver is up-regulated by lipopolysaccharide and lipoteichoic acid. *FEBS Lett* 412:511-514.

6. MacMicking, J.D., C. Nathan, G. Hom, N. Chartrain, D.S. Fletcher, M. Trumbauer, K. Stevens, Q.W. Xie, K. Sokol, N. Hutchinson, and et al. 1995. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* 81:641-650.

7. Salkowski, C.A., G. Detore, R. McNally, N. van Rooijen, and S.N. Vogel. 1997. Regulation of inducible nitric oxide synthase messenger RNA expression and nitric oxide production by lipopolysaccharide in vivo: the roles of macrophages, endogenous IFN-gamma, and TNF receptor-1-mediated signaling. *J Immunol* 158:905-912.

8. Whittle, B.J. 1995. Nitric oxide in physiology and pathology. *Histochem J* 27:727-737.

9. Opdenakker, G., S. Masure, B. Grillet, and J. Van Damme. 1991. Cytokine-mediated regulation of human leukocyte gelatinases and role in arthritis. *Lymphokine Cytokine Res* 10:317-324.

10. Masure, S., P. Proost, J. Van Damme, and G. Opdenakker. 1991. Purification and identification of 91-kDa neutrophil gelatinase. Release by the activating peptide interleukin-8. *Eur J Biochem* 198:391-398.

11. Pugin, J., M.C. Widmer, S. Kossodo, C.M. Liang, H.L.n. Preas, and A.F. Suffredini. 1999. Human neutrophils secrete gelatinase B in vitro and in vivo in response to endotoxin and proinflammatory mediators. *Am J Respir Cell Mol Biol* 20:458-464.

12. Yassen, K.A., H.F. Galley, and N.R. Webster. 2001. Matrix metalloproteinase-9 concentrations in critically ill patients. *Anaesthesia* 56:729-732.

13. Nakamura, T., I. Ebihara, N. Shimada, H. Shoji, and H. Koide. 1998. Modulation of plasma metalloproteinase-9 concentrations and peripheral

blood monocyte mRNA levels in patients with septic shock: effect of fiber-immobilized polymyxin B treatment. *Am J Med Sci* 316:355-360.

14. Dubois, B., S. Starckx, A. Pagenstecher, J. Oord, B. Arnold, and G. Opdenakker. 2002. Gelatinase B deficiency protects against endotoxin shock. *Eur J Immunol* 32:2163-2171.

15. Renckens, R., J.J. Roelofs, S. Florquin, A.F. de Vos, H.R. Lijnen, C. van't Veer, and T. van der Poll. 2006. Matrix metalloproteinase-9 deficiency impairs host defense against abdominal sepsis. *J Immunol* 176:3735-3741.

16. D'Haese, A., A. Wuyts, C. Dillen, B. Dubois, A. Billiau, H. Heremans, J. Van Damme, B. Arnold, and G. Opdenakker. 2000. In vivo neutrophil recruitment by granulocyte chemotactic protein-2 is assisted by gelatinase B/MMP-9 in the mouse. *J Interferon Cytokine Res* 20:667-674.

17. Hu, J., P.E. Van den Steen, C. Dillen, and G. Opdenakker. 2005. Targeting neutrophil collagenase/matrix metalloproteinase-8 and gelatinase B/matrix metalloproteinase-9 with a peptidomimetic inhibitor protects against endotoxin shock. *Biochem Pharmacol* 70:535-544.

18. Salter, H. 1882. On asthma: Its pathology and treatment. 284-285 pp.

19. Laitinen, L.A., M. Heino, A. Laitinen, T. Kava, and T. Haahtela. 1985. Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am Rev Respir Dis* 131:599-606.

20. Laitinen, A., L.A. Laitinen, and T. Haahtela. 1990. Bronchial biopsies in drug intervention studies in CB and COAD. *Agents Actions Suppl* 30:173-181.

21. Breeze, R.G., and E.B. Wheeldon. 1977. The cells of the pulmonary airways. *Am Rev Respir Dis* 116:705-777.

22. Murray, J.F., and J.A. Nadel. 1994. Respiratory Medicine. W.B. Sounders Company. 2711 pp.

23. Rhodin, J.A. 1966. The ciliated cell. Ultrastructure and function of the human tracheal mucosa. *Am Rev Respir Dis* 93:Suppl:1-15.

24. Staub, N.C. 1975. Some aspects of airways structure and function. *Postgrad Med J* 51:21-35.

25. Crapo, J.D., B.E. Barry, P. Gehr, M. Bachofen, and E.R. Weibel. 1982. Cell number and cell characteristics of the normal human lung. *Am Rev Respir Dis* 126:332-337.

26. Low, F.N. 1952. Electron microscopy of the rat lung. *Anat Rec* 113:437-449.

27. Williams, M.C. 2003. Alveolar type I cells: molecular phenotype and development. *Annu Rev Physiol* 65:669-695.

28. Evans, M.J., L.J. Cabral, R.J. Stephens, and G. Freeman. 1975. Transformation of alveolar type 2 cells to type 1 cells following exposure to NO2. *Exp Mol Pathol* 22:142-150.

29. Witherden, I.R., E.J. Vanden Bon, P. Goldstraw, C. Ratcliffe, U. Pastorino, and T.D. Tetley. 2004. Primary human alveolar type II epithelial cell chemokine release: effects of cigarette smoke and neutrophil elastase. *Am J Respir Cell Mol Biol* 30:500-509.

30. Clements, J.A., E.S. Brown, and R.P. Johnson. 1958. Pulmonary surface tension and the mucus lining of the lungs: some theoretical considerations. *J Appl Physiol* 12:262-268.

31. Gillette, J.R., D.C. Davis, and H.A. Sasame. 1972. Cytochrome P-450 and its role in drug metabolism. *Annu Rev Pharmacol* 12:57-84.

32. Jones, G.S., P.R. Miles, R.C. Lantz, D.E. Hinton, and V. Castranova. 1982. Ionic content and regulation of cellular volume in rat alveolar type II cells. *J Appl Physiol* 53:258-266.

33. Castranova, V., J. Rabovsky, J.H. Tucker, and P.R. Miles. 1988. The alveolar type II epithelial cell: a multifunctional pneumocyte. *Toxicol Appl Pharmacol* 93:472-483.

34. Wardlaw, S.A., T.H. March, and S.A. Belinsky. 2000. Cyclooxygenase-2 expression is abundant in alveolar type II cells in lung cancer-sensitive mouse strains and in premalignant lesions. *Carcinogenesis* 21:1371-1377.

35. Hammerschmidt, S., H. Kuhn, U. Sack, A. Schlenska, C. Gessner, A. Gillissen, and H. Wirtz. 2005. Mechanical stretch alters alveolar type II cell mediator release toward a proinflammatory pattern. *Am J Respir Cell Mol Biol* 33:203-210.

36. Toga, H., T. Tobe, Y. Ueda, G.H. Yang, K. Osanai, M. Ishigaki, H. Okazaki, S. Katsuda, K. Takahashi, and N. Ohya. 2001. Inducible nitric oxide synthase expression and nuclear factor-kappaB activation in alveolar type II cells in lung injury. *Exp Lung Res* 27:485-504.

37. Pardo, A., K. Ridge, B. Uhal, J.I. Sznajder, and M. Selman. 1997. Lung alveolar epithelial cells synthesize interstitial collagenase and gelatinases A and B in vitro. *Int J Biochem Cell Biol* 29:901-910.

38. Rosseau, S., J. Selhorst, K. Wiechmann, K. Leissner, U. Maus, K. Mayer, F. Grimminger, W. Seeger, and J. Lohmeyer. 2000. Monocyte migration through the alveolar epithelial barrier: adhesion molecule mechanisms and impact of chemokines. *J Immunol* 164:427-435.

39. Eghtesad, M., H.E. Jackson, and A.C. Cunningham. 2001. Primary human alveolar epithelial cells can elicit the transendothelial migration of CD14+ monocytes and CD3+ lymphocytes. *Immunology* 102:157-164.

40. Koyama, S., E. Sato, H. Nomura, K. Kubo, M. Miura, T. Yamashita, S. Nagai, and T. Izumi. 1999. Monocyte chemotactic factors released from type II pneumocyte-like cells in response to TNF-alpha and IL-1alpha. *Eur Respir J* 13:820-828.

41. Wolf, M., I. Clark-Lewis, C. Buri, H. Langen, M. Lis, and L. Mazzucchelli. 2003. Cathepsin D specifically cleaves the chemokines macrophage inflammatory protein-1 alpha, macrophage inflammatory protein-1 beta, and SLC that are expressed in human breast cancer. *Am J Pathol* 162:1183-1190.

42. McQuibban, G.A., G.S. Butler, J.H. Gong, L. Bendall, C. Power, I. Clark-Lewis, and C.M. Overall. 2001. Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1. *J Biol Chem* 276:43503-43508.

43. Van den Steen, P.E., P. Proost, A. Wuyts, J. Van Damme, and G. Opdenakker. 2000. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood* 96:2673-2681.

44. Betsuyaku, T., Y. Fukuda, W.C. Parks, J.M. Shipley, and R.M. Senior. 2000. Gelatinase B is required for alveolar bronchiolization after intratracheal bleomycin. *Am J Pathol* 157:525-535.

45. Lotvall, J., M. Inman, and P. O'Byrne. 1998. Measurement of airway hyperresponsiveness: new considerations. *Thorax* 53:419-424.

46. Jain, B., I. Rubinstein, R.A. Robbins, K.L. Leise, and J.H. Sisson. 1993. Modulation of airway epithelial cell ciliary beat frequency by nitric oxide. *Biochem Biophys Res Commun* 191:83-88.

47. Karupiah, G., Q.W. Xie, R.M. Buller, C. Nathan, C. Duarte, and J.D. MacMicking. 1993. Inhibition of viral replication by interferon-gamma-induced nitric oxide synthase. *Science* 261:1445-1448.

48. Denis, M. 1991. Interferon-gamma-treated murine macrophages inhibit growth of tubercle bacilli via the generation of reactive nitrogen intermediates. *Cell Immunol* 132:150-157.

49. Hibbs, J.B., Jr., R.R. Taintor, Z. Vavrin, and E.M. Rachlin. 1988. Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem Biophys Res Commun* 157:87-94.

50. Stretton, D. 1991. Non-adrenergic, non-cholinergic neural control of the airways. *Clin Exp Pharmacol Physiol* 18:675-684.

51. Belvisi, M.G., C.D. Stretton, M. Yacoub, and P.J. Barnes. 1992. Nitric oxide is the endogenous neurotransmitter of bronchodilator nerves in humans. *Eur J Pharmacol* 210:221-222.

52. Miura, M., H. Yamauchi, M. Ichinose, Y. Ohuchi, N. Kageyama, M. Tomaki, N. Endoh, and K. Shirato. 1997. Impairment of neural nitric oxide-mediated relaxation after antigen exposure in guinea pig airways in vitro. *Am J Respir Crit Care Med* 156:217-222.

53. Dupuy, P.M., S.A. Shore, J.M. Drazen, C. Frostell, W.A. Hill, and W.M. Zapol. 1992. Bronchodilator action of inhaled nitric oxide in guinea pigs. *J Clin Invest* 90:421-428.

54. Hogman, M., C.G. Frostell, H. Hedenstrom, and G. Hedenstierna. 1993. Inhalation of nitric oxide modulates adult human bronchial tone. *Am Rev Respir Dis* 148:1474-1478.

55. Gaston, B., S. Sears, J. Woods, J. Hunt, M. Ponaman, T. McMahon, and J.S. Stamler. 1998. Bronchodilator S-nitrosothiol deficiency in asthmatic respiratory failure. *Lancet* 351:1317-1319.

56. Gaston, B., J. Reilly, J.M. Drazen, J. Fackler, P. Ramdev, D. Arnelle, M.E. Mullins, D.J. Sugarbaker, C. Chee, D.J. Singel, and et al. 1993. Endogenous nitrogen oxides and bronchodilator S-nitrosothiols in human airways. *Proc Natl Acad Sci U S A* 90:10957-10961.

57. Gaston, B., J.M. Drazen, A. Jansen, D.A. Sugarbaker, J. Loscalzo, W. Richards, and J.S. Stamler. 1994. Relaxation of human bronchial smooth muscle by S-nitrosothiols in vitro. *J Pharmacol Exp Ther* 268:978-984.

58. Nijkamp, F.P., H.J. van der Linde, and G. Folkerts. 1993. Nitric oxide synthesis inhibitors induce airway hyperresponsiveness in the guinea pig in vivo and in vitro. Role of the epithelium. *Am Rev Respir Dis* 148:727-734.

59. Figini, M., F.L. Ricciardolo, P. Javdan, F.P. Nijkamp, C. Emanueli, P. Pradelles, G. Folkerts, and P. Geppetti. 1996. Evidence that epithelium-derived relaxing factor released by bradykinin in the guinea pig trachea is nitric oxide. *Am J Respir Crit Care Med* 153:918-923.

60. Filep, J.G., B. Battistini, and P. Sirois. 1993. Induction by endothelin-1 of epithelium-dependent relaxation of guinea-pig trachea in vitro: role for nitric oxide. *Br J Pharmacol* 109:637-644.

61. Emanueli, C., F. Ricciardolo, L. Vergnani, C. Bertrand, F. Ricci, N. Manzoli, G. Folkerts, F.P. Nijkamp, and P. Geppetti. 1998. Characterization of the endothelin receptor subtype mediating epithelium-derived relaxant nitric oxide release from guinea-pig trachea. *Br J Pharmacol* 125:963-968.

62. Figini, M., C. Emanueli, C. Bertrand, P. Javdan, and P. Geppetti. 1996. Evidence that tachykinins relax the guinea-pig trachea via nitric oxide release and by stimulation of a septide-insensitive NK1 receptor. *Br J Pharmacol* 117:1270-1276.

63. Ali, S., W.J. Metzger, H.A. Olanrewaju, and S.J. Mustafa. 1997. Adenosine receptor-mediated relaxation of rabbit airway smooth muscle: a role for nitric oxide. *Am J Physiol* 273:L581-587.

64. Ricciardolo, F.L., P. Geppetti, A. Mistretta, J.A. Nadel, M.A. Sapienza, S. Bellofiore, and G.U. Di Maria. 1996. Randomised double-blind placebo-

controlled study of the effect of inhibition of nitric oxide synthesis in bradykinin-induced asthma. *Lancet* 348:374-377.

65. Nagaki, M., M.N. Shimura, T. Irokawa, T. Sasaki, and K. Shirato. 1995. Nitric oxide regulation of glycoconjugate secretion from feline and human airways in vitro. *Respir Physiol* 102:89-95.

66. Adler, K.B., B.M. Fischer, H. Li, N.H. Choe, and D.T. Wright. 1995. Hypersecretion of mucin in response to inflammatory mediators by guinea pig tracheal epithelial cells in vitro is blocked by inhibition of nitric oxide synthase. *Am J Respir Cell Mol Biol* 13:526-530.

67. Jain, B., I. Rubinstein, R.A. Robbins, and J.H. Sisson. 1995. TNF-alpha and IL-1 beta upregulate nitric oxide-dependent ciliary motility in bovine airway epithelium. *Am J Physiol* 268:L911-917.

68. Duszyk, M. 2001. Regulation of anion secretion by nitric oxide in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 281:L450-457.

69. Kelly, E.A., and N.N. Jarjour. 2003. Role of matrix metalloproteinases in asthma. *Curr Opin Pulm Med* 9:28-33.

70. Suzuki, R., T. Kato, Y. Miyazaki, M. Iwata, Y. Noda, K. Takagi, N. Nakashima, and K. Torii. 2001. Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in sputum from patients with bronchial asthma. *J Asthma* 38:477-484.

71. Lemjabbar, H., P. Gosset, C. Lamblin, I. Tillie, D. Hartmann, B. Wallaert, A.B. Tonnel, and C. Lafuma. 1999. Contribution of 92 kDa gelatinase/type IV

collagenase in bronchial inflammation during status asthmaticus. *Am J Respir Crit Care Med* 159:1298-1307.

72. Yao, P.M., H. Lemjabbar, M.P. D'Ortho, B. Maitre, P. Gossett, B. Wallaert, and C. Lafuma. 1999. Balance between MMP-9 and TIMP-1 expressed by human bronchial epithelial cells: relevance to asthma. *Ann N Y Acad Sci* 878:512-514.

73. Zuo, F., N. Kaminski, E. Eugui, J. Allard, Z. Yakhini, A. Ben-Dor, L. Lollini, D. Morris, Y. Kim, B. DeLustro, D. Sheppard, A. Pardo, M. Selman, and R.A. Heller. 2002. Gene expression analysis reveals matrilysin as a key regulator of pulmonary fibrosis in mice and humans. *Proc Natl Acad Sci U S A* 99:6292-6297.

74. Pardo, A., R. Barrios, V. Maldonado, J. Melendez, J. Perez, V. Ruiz, L. Segura-Valdez, J.I. Sznajder, and M. Selman. 1998. Gelatinases A and B are up-regulated in rat lungs by subacute hyperoxia: pathogenetic implications. *Am J Pathol* 153:833-844.

75. Dik, W.A., A.H. van Kaam, T. Dekker, B.A. Naber, D.J. Janssen, A.A. Kroon, L.J. Zimmermann, M.A. Versnel, and R. Lutter. 2006. Early increased levels of matrix metalloproteinase-9 in neonates recovering from respiratory distress syndrome. *Biol Neonate* 89:6-14.

76. Sternlicht, M.D., and Z. Werb. 2001. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 17:463-516.

77. Kenagy, R.D., and A.W. Clowes. 1994. A possible role for MMP-2 and MMP-9 in the migration of primate arterial smooth muscle cells through native matrix. *Ann N Y Acad Sci* 732:462-465.

78. Schnaper, H.W., D.S. Grant, W.G. Stetler-Stevenson, R. Fridman, G. D'Orazi, A.N. Murphy, R.E. Bird, M. Hoythya, T.R. Fuerst, D.L. French, and et al. 1993. Type IV collagenase(s) and TIMPs modulate endothelial cell morphogenesis in vitro. *J Cell Physiol* 156:235-246.

79. Welgus, H.G., E.J. Campbell, J.D. Cury, A.Z. Eisen, R.M. Senior, S.M. Wilhelm, and G.I. Goldberg. 1990. Neutral metalloproteinases produced by human mononuclear phagocytes. Enzyme profile, regulation, and expression during cellular development. *J Clin Invest* 86:1496-1502.

80. Ohno, I., H. Ohtani, Y. Nitta, J. Suzuki, H. Hoshi, M. Honma, S. Isoyama, Y. Tanno, G. Tamura, K. Yamauchi, H. Nagura, and K. Shirato. 1997. Eosinophils as a source of matrix metalloproteinase-9 in asthmatic airway inflammation. *Am J Respir Cell Mol Biol* 16:212-219.

81. Kanbe, N., A. Tanaka, M. Kanbe, A. Itakura, M. Kurosawa, and H. Matsuda. 1999. Human mast cells produce matrix metalloproteinase 9. *Eur J Immunol* 29:2645-2649.

82. Weeks, B.S., H.W. Schnaper, M. Handy, E. Holloway, and H.K. Kleinman. 1993. Human T lymphocytes synthesize the 92 kDa type IV collagenase (gelatinase B). *J Cell Physiol* 157:644-649.

83. Vignola, A.M., L. Riccobono, A. Mirabella, M. Profita, P. Chanez, V. Bellia, G. Mautino, P. D'Accardi, J. Bousquet, and G. Bonsignore. 1998. Sputum

metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio correlates with airflow obstruction in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 158:1945-1950.

84. Mautino, G., N. Oliver, P. Chanez, J. Bousquet, and F. Capony. 1997. Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid and by alveolar macrophages of asthmatics. *Am J Respir Cell Mol Biol* 17:583-591.

85. Cataldo, D., C. Munaut, A. Noel, F. Frankenne, P. Bartsch, J.M. Foidart, and R. Louis. 2001. Matrix metalloproteinases and TIMP-1 production by peripheral blood granulocytes from COPD patients and asthmatics. *Allergy* 56:145-151.

86. Hoshino, M., Y. Nakamura, J. Sim, J. Shimojo, and S. Isogai. 1998. Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *J Allergy Clin Immunol* 102:783-788.

87. Lee, Y.C., H.B. Lee, Y.K. Rhee, and C.H. Song. 2001. The involvement of matrix metalloproteinase-9 in airway inflammation of patients with acute asthma. *Clin Exp Allergy* 31:1623-1630.

88. Tanaka, H., N. Miyazaki, K. Oashi, S. Tanaka, M. Ohmichi, and S. Abe. 2000. Sputum matrix metalloproteinase-9: tissue inhibitor of metalloproteinase-1 ratio in acute asthma. *J Allergy Clin Immunol* 105:900-905.

89. Daniels, S.E., S. Bhattacharrya, A. James, N.I. Leaves, A. Young, M.R. Hill, J.A. Faux, G.F. Ryan, P.N. le Souef, G.M. Lathrop, A.W. Musk, and W.O.

Cookson. 1996. A genome-wide search for quantitative trait loci underlying asthma. *Nature* 383:247-250.

90. Trifilieff, A., C. Walker, T. Keller, G. Kottirsch, and U. Neumann. 2002. Pharmacological profile of PKF242-484 and PKF241-466, novel dual inhibitors of TNF-alpha converting enzyme and matrix metalloproteinases, in models of airway inflammation. *Br J Pharmacol* 135:1655-1664.

91. Lee, Y.C., C.H. Song, H.B. Lee, J.L. Oh, Y.K. Rhee, H.S. Park, and G.Y. Koh. 2001. A murine model of toluene diisocyanate-induced asthma can be treated with matrix metalloproteinase inhibitor. *J Allergy Clin Immunol* 108:1021-1026.

92. Cataldo, D.D., K.G. Tournoy, K. Vermaelen, C. Munaut, J.M. Foidart, R. Louis, A. Noel, and R.A. Pauwels. 2002. Matrix metalloproteinase-9 deficiency impairs cellular infiltration and bronchial hyperresponsiveness during allergen-induced airway inflammation. *Am J Pathol* 161:491-498.

93. Vermaelen, K.Y., D. Cataldo, K. Tournoy, T. Maes, A. Dhulst, R. Louis, J.M. Foidart, A. Noel, and R. Pauwels. 2003. Matrix metalloproteinase-9-mediated dendritic cell recruitment into the airways is a critical step in a mouse model of asthma. *J Immunol* 171:1016-1022.

94. Ichiyasu, H., J.M. McCormack, K.M. McCarthy, D. Dombkowski, F.I. Preffer, and E.E. Schneeberger. 2004. Matrix metalloproteinase-9-deficient dendritic cells have impaired migration through tracheal epithelial tight junctions. *Am J Respir Cell Mol Biol* 30:761-770.

95. McMillan, S.J., J. Kearley, J.D. Campbell, X.W. Zhu, K.Y. Larbi, J.M. Shipley, R.M. Senior, S. Nourshargh, and C.M. Lloyd. 2004. Matrix metalloproteinase-9 deficiency results in enhanced allergen-induced airway inflammation. *J Immunol* 172:2586-2594.

96. Lee, C.G., R.J. Homer, Z. Zhu, S. Lanone, X. Wang, V. Koteliansky, J.M. Shipley, P. Gotwals, P. Noble, Q. Chen, R.M. Senior, and J.A. Elias. 2001. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). *J Exp Med* 194:809-821.

97. Kurzrok, k., and C. Lieb. 1930. Biochemical studies of human semen. II. The action of semen on the human uterus. *Proc Soc. Exp. Biol* 28:268-272.

98. Miyamoto, T., N. Ogino, S. Yamamoto, and O. Hayaishi. 1976. Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J Biol Chem* 251:2629-2636.

99. Xie, W.L., J.G. Chipman, D.L. Robertson, R.L. Erikson, and D.L. Simmons. 1991. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc Natl Acad Sci U S A* 88:2692-2696.

100. Kujubu, D.A., B.S. Fletcher, B.C. Varnum, R.W. Lim, and H.R. Herschman. 1991. TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J Biol Chem* 266:12866-12872.

101. Vane, J.R. 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* 231:232-235.

102. Chandrasekharan, N.V., H. Dai, K.L. Roos, N.K. Evanson, J. Tomsik, T.S. Elton, and D.L. Simmons. 2002. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proc Natl Acad Sci U S A* 99:13926-13931.

103. Kis, B., J.A. Snipes, and D.W. Busija. 2005. Acetaminophen and the cyclooxygenase-3 puzzle: sorting out facts, fictions, and uncertainties. *J Pharmacol Exp Ther* 315:1-7.

104. Landino, L.M., B.C. Crews, M.D. Timmons, J.D. Morrow, and L.J. Marnett. 1996. Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc Natl Acad Sci U S A* 93:15069-15074.

105. Goodwin, D.C., L.M. Landino, and L.J. Marnett. 1999. Effects of nitric oxide and nitric oxide-derived species on prostaglandin endoperoxide synthase and prostaglandin biosynthesis. *Faseb J* 13:1121-1136.

106. Hemler, M.E., G. Graff, and W.E. Lands. 1978. Accelerative autoactivation of prostaglandin biosynthesis by PGG2. *Biochem Biophys Res Commun* 85:1325-1331.

107. Hemler, M.E., H.W. Cook, and W.E. Lands. 1979. Prostaglandin biosynthesis can be triggered by lipid peroxides. *Arch Biochem Biophys* 193:340-345.

108. Yu, M., D. Ives, and C.S. Ramesha. 1997. Synthesis of prostaglandin E2 ethanolamide from anandamide by cyclooxygenase-2. *J Biol Chem* 272:21181-21186.

109. Kozak, K.R., S.W. Rowlinson, and L.J. Marnett. 2000. Oxygenation of the endocannabinoid, 2-arachidonylglycerol, to glyceryl prostaglandins by cyclooxygenase-2. *J Biol Chem* 275:33744-33749.

110. Barnes, P.J., K.F. Chung, and C.P. Page. 1998. Inflammatory mediators of asthma: an update. *Pharmacol Rev* 50:515-596.

111. Dubois, R.N., S.B. Abramson, L. Crofford, R.A. Gupta, L.S. Simon, L.B. Van De Putte, and P.E. Lipsky. 1998. Cyclooxygenase in biology and disease. *Faseb J* 12:1063-1073.

112. Demoly, P., D. Jaffuel, N. Lequeux, B. Weksler, C. Creminon, F.B. Michel, P. Godard, and J. Bousquet. 1997. Prostaglandin H synthase 1 and 2 immunoreactivities in the bronchial mucosa of asthmatics. *Am J Respir Crit Care Med* 155:670-675.

113. Schildknecht, S., M. Bachschmid, A. Baumann, and V. Ullrich. 2004. COX-2 inhibitors selectively block prostacyclin synthesis in endotoxin-exposed vascular smooth muscle cells. *Faseb J* 18:757-759.

114. Claria, J. 2003. Cyclooxygenase-2 biology. *Curr Pharm Des* 9:2177-2190.

115. Primakoff, P., and D.G. Myles. 2000. The ADAM gene family: surface proteins with adhesion and protease activity. *Trends Genet* 16:83-87.

116. Strongin, A.Y., I. Collier, G. Bannikov, B.L. Marmer, G.A. Grant, and G.I. Goldberg. 1995. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. *J Biol Chem* 270:5331-5338.

117. Empey, D.W., L.A. Laitinen, L. Jacobs, W.M. Gold, and J.A. Nadel. 1976. Mechanisms of bronchial hyperreactivity in normal subjects after upper respiratory tract infection. *Am Rev Respir Dis* 113:131-139.

118. Wilhelm, S.M., I.E. Collier, B.L. Marmer, A.Z. Eisen, G.A. Grant, and G.I. Goldberg. 1989. SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. *J Biol Chem* 264:17213-17221.

119. Devarajan, P., J.J. Johnston, S.S. Ginsberg, H.E. Van Wart, and N. Berliner. 1992. Structure and expression of neutrophil gelatinase cDNA. Identity with type IV collagenase from HT1080 cells. *J Biol Chem* 267:25228-25232.

120. Shipley, J.M., G.A. Doyle, C.J. Fliszar, Q.Z. Ye, L.L. Johnson, S.D. Shapiro, H.G. Welgus, and R.M. Senior. 1996. The structural basis for the elastolytic activity of the 92-kDa and 72-kDa gelatinases. Role of the fibronectin type II-like repeats. *J Biol Chem* 271:4335-4341.

121. Van Wart, H.E., and H. Birkedal-Hansen. 1990. The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc Natl Acad Sci U S A* 87:5578-5582.

122. Kobayashi, T., J. Kishimoto, Y. Ge, W. Jin, D.L. Hudson, N. Ouahes, R. Ehama, H. Shinkai, and R.E. Burgeson. 2001. A novel mechanism of matrix metalloproteinase-9 gene expression implies a role for keratinization. *EMBO Rep* 2:604-608.

123. Sato, H., and M. Seiki. 1993. Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. *Oncogene* 8:395-405.

124. Yokoo, T., and M. Kitamura. 1996. Dual regulation of IL-1 beta-mediated matrix metalloproteinase-9 expression in mesangial cells by NF-kappa B and AP-1. *Am J Physiol* 270:F123-130.

125. Van den Steen, P.E., B. Dubois, I. Nelissen, P.M. Rudd, R.A. Dwek, and G. Opdenakker. 2002. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol* 37:375-536.

126. Zhang, B., S. Ye, S.M. Herrmann, P. Eriksson, M. de Maat, A. Evans, D. Arveiler, G. Luc, F. Cambien, A. Hamsten, H. Watkins, and A.M. Henney. 1999. Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis. *Circulation* 99:1788-1794.

127. Gu, Z., M. Kaul, B. Yan, S.J. Kridel, J. Cui, A. Strongin, J.W. Smith, R.C. Liddington, and S.A. Lipton. 2002. S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science* 297:1186-1190.

128. van der Vliet, A., C.A. O'Neill, C.E. Cross, J.M. Koostra, W.G. Volz, B. Halliwell, and S. Louie. 1999. Determination of low-molecular-mass antioxidant concentrations in human respiratory tract lining fluids. *Am J Physiol* 276:L289-296.

129. Sies, H. 1999. Glutathione and its role in cellular functions. *Free Radic Biol Med* 27:916-921.

130. Okamoto, T., T. Akaike, T. Sawa, Y. Miyamoto, A. van der Vliet, and H. Maeda. 2001. Activation of matrix metalloproteinases by peroxynitrite-induced protein S-glutathiolation via disulfide S-oxide formation. *J Biol Chem* 276:29596-29602.

131. Okamoto, T., T. Akuta, F. Tamura, A. van Der Vliet, and T. Akaike. 2004. Molecular mechanism for activation and regulation of matrix metalloproteinases during bacterial infections and respiratory inflammation. *Biol Chem* 385:997-1006.

132. Frears, E.R., Z. Zhang, D.R. Blake, J.P. O'Connell, and P.G. Winyard. 1996. Inactivation of tissue inhibitor of metalloproteinase-1 by peroxynitrite. *FEBS Lett* 381:21-24.

133. Janssen-Heininger, Y.M., M.E. Poynter, and P.A. Baeuerle. 2000. Recent advances towards understanding redox mechanisms in the activation of nuclear factor kappaB. *Free Radic Biol Med* 28:1317-1327.

134. Akool el, S., H. Kleinert, F.M. Hamada, M.H. Abdelwahab, U. Forstermann, J. Pfeilschifter, and W. Eberhardt. 2003. Nitric oxide increases the decay of matrix metalloproteinase 9 mRNA by inhibiting the expression of mRNA-stabilizing factor HuR. *Mol Cell Biol* 23:4901-4916.

135. Ishii, Y., T. Ogura, M. Tatemichi, H. Fujisawa, F. Otsuka, and H. Esumi. 2003. Induction of matrix metalloproteinase gene transcription by nitric oxide and mechanisms of MMP-1 gene induction in human melanoma cell lines. *Int J Cancer* 103:161-168.

136. Fukuda, Y., M. Ishizaki, Y. Okada, M. Seiki, and N. Yamanaka. 2000. Matrix metalloproteinases and tissue inhibitor of metalloproteinase-2 in fetal rabbit lung. *Am J Physiol Lung Cell Mol Physiol* 279:L555-561.

137. Atkinson, J.J., and R.M. Senior. 2003. Matrix metalloproteinase-9 in lung remodeling. *Am J Respir Cell Mol Biol* 28:12-24.

138. Egeblad, M., and Z. Werb. 2002. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2:161-174.

139. Herman, M.P., G.K. Sukhova, W. Kisiel, D. Foster, M.R. Kehry, P. Libby, and U. Schonbeck. 2001. Tissue factor pathway inhibitor-2 is a novel inhibitor of matrix metalloproteinases with implications for atherosclerosis. *J Clin Invest* 107:1117-1126.

140. Murphy, G., E.C. Cartwright, A. Sellers, and J.J. Reynolds. 1977. The detection and characterisation of collagenase inhibitors from rabbit tissues in culture. *Biochim Biophys Acta* 483:493-498.

141. Goldberg, G.I., A. Strongin, I.E. Collier, L.T. Genrich, and B.L. Marmer. 1992. Interaction of 92-kDa type IV collagenase with the tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial collagenase, and activation of the proenzyme with stromelysin. *J Biol Chem* 267:4583-4591.

142. MacMicking, J., Q.W. Xie, and C. Nathan. 1997. Nitric oxide and macrophage function. *Annu Rev Immunol* 15:323-350.

143. Nathan, C., and Q.W. Xie. 1994. Regulation of biosynthesis of nitric oxide. *J Biol Chem* 269:13725-13728.

144. Hevel, J.M., K.A. White, and M.A. Marletta. 1991. Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. *J Biol Chem* 266:22789-22791.

145. Stuehr, D.J., H.J. Cho, N.S. Kwon, M.F. Weise, and C.F. Nathan. 1991. Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc Natl Acad Sci U S A* 88:7773-7777.

146. Bogdan, C., Y. Vodovotz, J. Paik, Q.W. Xie, and C. Nathan. 1994. Mechanism of suppression of nitric oxide synthase expression by interleukin-4 in primary mouse macrophages. *J Leukoc Biol* 55:227-233.

147. McCall, T.B., R.M. Palmer, and S. Moncada. 1992. Interleukin-8 inhibits the induction of nitric oxide synthase in rat peritoneal neutrophils. *Biochem Biophys Res Commun* 186:680-685.

148. Cunha, F.Q., S. Moncada, and F.Y. Liew. 1992. Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon-gamma in murine macrophages. *Biochem Biophys Res Commun* 182:1155-1159.

149. Mulsch, A., B. Schray-Utz, P.I. Mordvintcev, S. Hauschildt, and R. Busse. 1993. Diethyldithiocarbamate inhibits induction of macrophage NO synthase. *FEBS Lett* 321:215-218.

150. Sherman, M.P., E.E. Aeberhard, V.Z. Wong, J.M. Griscavage, and L.J. Ignarro. 1993. Pyrrolidine dithiocarbamate inhibits induction of nitric oxide synthase activity in rat alveolar macrophages. *Biochem Biophys Res Commun* 191:1301-1308.

151. Dong, Z., X. Qi, K. Xie, and I.J. Fidler. 1993. Protein tyrosine kinase inhibitors decrease induction of nitric oxide synthase activity in lipopolysaccharide-responsive and lipopolysaccharide-nonresponsive murine macrophages. *J Immunol* 151:2717-2724.

152. Radomski, M.W., R.M. Palmer, and S. Moncada. 1990. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc Natl Acad Sci U S A* 87:10043-10047.

153. Kelm, M. 1999. Nitric oxide metabolism and breakdown. *Biochim Biophys Acta* 1411:273-289.

154. Garthwaite, J., and C.L. Boulton. 1995. Nitric oxide signaling in the central nervous system. *Annu Rev Physiol* 57:683-706.

155. Moncada, S., and A. Higgs. 1993. The L-arginine-nitric oxide pathway. *N Engl J Med* 329:2002-2012.

156. Lohmann, S.M., A.B. Vaandrager, A. Smolenski, U. Walter, and H.R. De Jonge. 1997. Distinct and specific functions of cGMP-dependent protein kinases. *Trends Biochem Sci* 22:307-312.

157. Degerman, E., P. Belfrage, and V.C. Manganiello. 1997. Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3). *J Biol Chem* 272:6823-6826.

158. Zagotta, W.N., and S.A. Siegelbaum. 1996. Structure and function of cyclic nucleotide-gated channels. *Annu Rev Neurosci* 19:235-263.

159. Torgersen, K.M., T. Vang, H. Abrahamsen, S. Yaqub, and K. Tasken. 2002. Molecular mechanisms for protein kinase A-mediated modulation of immune function. *Cell Signal* 14:1-9.

160. Matsunaga, E. 1981. Genetics of Wilms' tumor. *Hum Genet* 57:231-246.

161. Call, K.M., T. Glaser, C.Y. Ito, A.J. Buckler, J. Pelletier, D.A. Haber, E.A. Rose, A. Kral, H. Yeger, W.H. Lewis, and et al. 1990. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell* 60:509-520.

162. Haber, D.A., A.J. Buckler, T. Glaser, K.M. Call, J. Pelletier, R.L. Sohn, E.C. Douglass, and D.E. Housman. 1990. An internal deletion within an 11p13 zinc finger gene contributes to the development of Wilms' tumor. *Cell* 61:1257-1269.

163. Rose, E.A., T. Glaser, C. Jones, C.L. Smith, W.H. Lewis, K.M. Call, M. Minden, E. Champagne, L. Bonetta, H. Yeger, and et al. 1990. Complete physical map of the WAGR region of 11p13 localizes a candidate Wilms' tumor gene. *Cell* 60:495-508.

164. Menke, A.L., and A. Schedl. 2003. WT1 and glomerular function. *Semin Cell Dev Biol* 14:233-240.

165. Coppes, M.J., G.J. Liefers, P. Paul, H. Yeger, and B.R. Williams. 1993. Homozygous somatic Wt1 point mutations in sporadic unilateral Wilms tumor. *Proc Natl Acad Sci U S A* 90:1416-1419.

166. Cowell, J.K., R.B. Wadey, D.A. Haber, K.M. Call, D.E. Housman, and J. Pritchard. 1991. Structural rearrangements of the WT1 gene in Wilms' tumour cells. *Oncogene* 6:595-599.

167. Varanasi, R., N. Bardeesy, M. Ghahremani, M.J. Petruzzi, N. Nowak, M.A. Adam, P. Grundy, T.B. Shows, and J. Pelletier. 1994. Fine structure analysis of the WT1 gene in sporadic Wilms tumors. *Proc Natl Acad Sci U S A* 91:3554-3558.

168. Discenza, M.T., and J. Pelletier. 2004. Insights into the physiological role of WT1 from studies of genetically modified mice. *Physiol Genomics* 16:287-300.

169. Haber, D.A., R.L. Sohn, A.J. Buckler, J. Pelletier, K.M. Call, and D.E. Housman. 1991. Alternative splicing and genomic structure of the Wilms tumor gene WT1. *Proc Natl Acad Sci U S A* 88:9618-9622.

170. Kim, J., K. Lee, and J. Pelletier. 1998. The DNA binding domains of the WT1 tumor suppressor gene product and chimeric EWS/WT1 oncoprotein are functionally distinct. *Oncogene* 16:1021-1030.

171. Bruening, W., and J. Pelletier. 1996. A non-AUG translational initiation event generates novel WT1 isoforms. *J Biol Chem* 271:8646-8654.

172. Scharnhorst, V., P. Dekker, A.J. van der Eb, and A.G. Jochemsen. 1999. Internal translation initiation generates novel WT1 protein isoforms with distinct biological properties. *J Biol Chem* 274:23456-23462.

173. Dallosso, A.R., A.L. Hancock, K.W. Brown, A.C. Williams, S. Jackson, and K. Malik. 2004. Genomic imprinting at the WT1 gene involves a novel coding

transcript (AWT1) that shows deregulation in Wilms' tumours. *Hum Mol Genet* 13:405-415.

174. Wang, Z.Y., Q.Q. Qiu, M. Gurrieri, J. Huang, and T.F. Deuel. 1995. WT1, the Wilms' tumor suppressor gene product, represses transcription through an interactive nuclear protein. *Oncogene* 10:1243-1247.

175. Wang, Z.Y., Q.Q. Qiu, K.T. Enger, and T.F. Deuel. 1993. A second transcriptionally active DNA-binding site for the Wilms tumor gene product, WT1. *Proc Natl Acad Sci U S A* 90:8896-8900.

176. Carpenter, B., K.J. Hill, M. Charalambous, K.J. Wagner, D. Lahiri, D.I. James, J.S. Andersen, V. Schumacher, B. Royer-Pokora, M. Mann, A. Ward, and S.G. Roberts. 2004. BASP1 is a transcriptional cosuppressor for the Wilms' tumor suppressor protein WT1. *Mol Cell Biol* 24:537-549.

177. Srichai, M.B., M. Konieczkowski, A. Padiyar, D.J. Konieczkowski, A. Mukherjee, P.S. Hayden, S. Kamat, M.A. El-Meanawy, S. Khan, P. Mundel, S.B. Lee, L.A. Bruggeman, J.R. Schelling, and J.R. Sedor. 2004. A WT1 co-regulator controls podocyte phenotype by shuttling between adhesion structures and nucleus. *J Biol Chem* 279:14398-14408.

178. Rico, M., A. Mukherjee, M. Konieczkowski, L.A. Bruggeman, R.T. Miller, S. Khan, J.R. Schelling, and J.R. Sedor. 2005. WT1-interacting protein and ZO-1 translocate into podocyte nuclei after puromycin aminonucleoside treatment. *Am J Physiol Renal Physiol* 289:F431-441.

179. Lee, T.H., S. Lwu, J. Kim, and J. Pelletier. 2002. Inhibition of Wilms tumor 1 transactivation by bone marrow zinc finger 2, a novel transcriptional repressor. *J Biol Chem* 277:44826-44837.

180. Maheswaran, S., S. Park, A. Bernard, J.F. Morris, F.J. Rauscher, 3rd, D.E. Hill, and D.A. Haber. 1993. Physical and functional interaction between WT1 and p53 proteins. *Proc Natl Acad Sci U S A* 90:5100-5104.

181. Maheswaran, S., C. Englert, P. Bennett, G. Heinrich, and D.A. Haber. 1995. The WT1 gene product stabilizes p53 and inhibits p53-mediated apoptosis. *Genes Dev* 9:2143-2156.

182. Holmes, G., S. Boterashvili, M. English, B. Wainwright, J. Licht, and M. Little. 1997. Two N-terminal self-association domains are required for the dominant negative transcriptional activity of WT1 Denys-Drash mutant proteins. *Biochem Biophys Res Commun* 233:723-728.

183. Moffett, P., W. Bruening, H. Nakagama, N. Bardeesy, D. Housman, D.E. Housman, and J. Pelletier. 1995. Antagonism of WT1 activity by protein self-association. *Proc Natl Acad Sci U S A* 92:11105-11109.

184. Reddy, J.C., J.C. Morris, J. Wang, M.A. English, D.A. Haber, Y. Shi, and J.D. Licht. 1995. WT1-mediated transcriptional activation is inhibited by dominant negative mutant proteins. *J Biol Chem* 270:10878-10884.

185. Nachtigal, M.W., Y. Hirokawa, D.L. Enyeart-VanHouten, J.N. Flanagan, G.D. Hammer, and H.A. Ingraham. 1998. Wilms' tumor 1 and Dax-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression. *Cell* 93:445-454.

186. Du, X., P. Hublitz, T. Gunther, D. Wilhelm, C. Englert, and R. Schule. 2002. The LIM-only coactivator FHL2 modulates WT1 transcriptional activity during gonadal differentiation. *Biochim Biophys Acta* 1577:93-101.

187. Eberle, D., B. Hegarty, P. Bossard, P. Ferre, and F. Foufelle. 2004. SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie* 86:839-848.

188. Rae, F.K., G. Martinez, K.R. Gillinder, A. Smith, G. Shooter, A.R. Forrest, S.M. Grimmond, and M.H. Little. 2004. Anlaysis of complementary expression profiles following WT1 induction versus repression reveals the cholesterol/fatty acid synthetic pathways as a possible major target of WT1. *Oncogene* 23:3067-3079.

189. Maheswaran, S., C. Englert, G. Zheng, S.B. Lee, J. Wong, D.P. Harkin, J. Bean, R. Ezzell, A.J. Garvin, R.T. McCluskey, J.A. DeCaprio, and D.A. Haber. 1998. Inhibition of cellular proliferation by the Wilms tumor suppressor WT1 requires association with the inducible chaperone Hsp70. *Genes Dev* 12:1108-1120.

190. Scharnhorst, V., A.J. van der Eb, and A.G. Jochemsen. 2001. WT1 proteins: functions in growth and differentiation. *Gene* 273:141-161.

191. Caricasole, A., A. Duarte, S.H. Larsson, N.D. Hastie, M. Little, G. Holmes, I. Todorov, and A. Ward. 1996. RNA binding by the Wilms tumor suppressor zinc finger proteins. *Proc Natl Acad Sci U S A* 93:7562-7566.

192. Bardeesy, N., and J. Pelletier. 1998. Overlapping RNA and DNA binding domains of the wt1 tumor suppressor gene product. *Nucleic Acids Res* 26:1784-1792.

193. Ladomery, M.R., J. Slight, S. Mc Ghee, and N.D. Hastie. 1999. Presence of WT1, the Wilm's tumor suppressor gene product, in nuclear poly(A)(+) ribonucleoprotein. *J Biol Chem* 274:36520-36526.

194. Davies, R.C., C. Calvio, E. Bratt, S.H. Larsson, A.I. Lamond, and N.D. Hastie. 1998. WT1 interacts with the splicing factor U2AF65 in an isoform-dependent manner and can be incorporated into spliceosomes. *Genes Dev* 12:3217-3225.

195. Kennedy, D., T. Ramsdale, J. Mattick, and M. Little. 1996. An RNA recognition motif in Wilms' tumour protein (WT1) revealed by structural modelling. *Nat Genet* 12:329-331.

196. Smolen, G.A., M.T. Vassileva, J. Wells, M.J. Matunis, and D.A. Haber. 2004. SUMO-1 modification of the Wilms' tumor suppressor WT1. *Cancer Res* 64:7846-7851.

197. Goodson, M.L., Y. Hong, R. Rogers, M.J. Matunis, O.K. Park-Sarge, and K.D. Sarge. 2001. Sumo-1 modification regulates the DNA binding activity of heat shock transcription factor 2, a promyelocytic leukemia nuclear body associated transcription factor. *J Biol Chem* 276:18513-18518.

198. Muller, S., M. Berger, F. Lehembre, J.S. Seeler, Y. Haupt, and A. Dejean. 2000. c-Jun and p53 activity is modulated by SUMO-1 modification. *J Biol Chem* 275:13321-13329.

199. Bies, J., J. Markus, and L. Wolff. 2002. Covalent attachment of the SUMO-1 protein to the negative regulatory domain of the c-Myb transcription factor modifies its stability and transactivation capacity. *J Biol Chem* 277:8999-9009.

200. Niksic, M., J. Slight, J.R. Sanford, J.F. Caceres, and N.D. Hastie. 2004. The Wilms' tumour protein (WT1) shuttles between nucleus and cytoplasm and is present in functional polysomes. *Hum Mol Genet* 13:463-471.

201. Sakamoto, Y., M. Yoshida, K. Semba, and T. Hunter. 1997. Inhibition of the DNA-binding and transcriptional repression activity of the Wilms' tumor gene product, WT1, by cAMP-dependent protein kinase-mediated phosphorylation of Ser-365 and Ser-393 in the zinc finger domain. *Oncogene* 15:2001-2012.

202. Ye, Y., B. Raychaudhuri, A. Gurney, C.E. Campbell, and B.R. Williams. 1996. Regulation of WT1 by phosphorylation: inhibition of DNA binding, alteration of transcriptional activity and cellular translocation. *Embo J* 15:5606-5615.

203. Vajjhala, P.R., E. Macmillan, T. Gonda, and M. Little. 2003. The Wilms' tumour suppressor protein, WT1, undergoes CRM1-independent nucleocytoplasmic shuttling. *FEBS Lett* 554:143-148.

204. Bickmore, W.A., K. Oghene, M.H. Little, A. Seawright, V. van Heyningen, and N.D. Hastie. 1992. Modulation of DNA binding specificity by alternative splicing of the Wilms tumor wt1 gene transcript. *Science* 257:235-237.

205. Rauscher, F.J., 3rd, J.F. Morris, O.E. Tournay, D.M. Cook, and T. Curran. 1990. Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. *Science* 250:1259-1262.

206. Epplen, J.T., A. Kyas, and W. Maueler. 1996. Genomic simple repetitive DNAs are targets for differential binding of nuclear proteins. *FEBS Lett* 389:92-95.

207. Maueler, W., G. Frank, M. Muller, and J.T. Epplen. 1994. A complex composed of at least two HeLa nuclear proteins protects preferentially one DNA strand of the simple (gt)n(ga)m containing region of intron 2 in HLA-DRB genes. *J Cell Biochem* 56:74-85.

208. Menke, A.L., A.J. van der Eb, and A.G. Jochemsen. 1998. The Wilms' tumor 1 gene: oncogene or tumor suppressor gene? *Int Rev Cytol* 181:151-212.

209. Nakagama, H., G. Heinrich, J. Pelletier, and D.E. Housman. 1995. Sequence and structural requirements for high-affinity DNA binding by the WT1 gene product. *Mol Cell Biol* 15:1489-1498.

210. Adachi, Y., S. Matsubara, C. Pedraza, M. Ozawa, J. Tsutsui, H. Takamatsu, H. Noguchi, T. Akiyama, and T. Muramatsu. 1996. Midkine as a novel target gene for the Wilms' tumor suppressor gene (WT1). *Oncogene* 13:2197-2203.

211. Mundlos, S., J. Pelletier, A. Darveau, M. Bachmann, A. Winterpacht, and B. Zabel. 1993. Nuclear localization of the protein encoded by the Wilms' tumor gene WT1 in embryonic and adult tissues. *Development* 119:1329-1341.

212. Wagner, N., K.D. Wagner, A. Hammes, K.M. Kirschner, V.P. Vidal, A. Schedl, and H. Scholz. 2005. A splice variant of the Wilms' tumour suppressor Wt1 is required for normal development of the olfactory system. *Development* 132:1327-1336.

213. Silberstein, G.B., K. Van Horn, P. Strickland, C.T. Roberts, Jr., and C.W. Daniel. 1997. Altered expression of the WT1 wilms tumor suppressor gene in human breast cancer. *Proc Natl Acad Sci U S A* 94:8132-8137.

214. Walker, C., F. Rutten, X. Yuan, H. Pass, D.M. Mew, and J. Everitt. 1994. Wilms' tumor suppressor gene expression in rat and human mesothelioma. *Cancer Res* 54:3101-3106.

215. Sharma, P.M., X. Yang, M. Bowman, V. Roberts, and S. Sukumar. 1992. Molecular cloning of rat Wilms' tumor complementary DNA and a study of messenger RNA expression in the urogenital system and the brain. *Cancer Res* 52:6407-6412.

216. Buckler, A.J., J. Pelletier, D.A. Haber, T. Glaser, and D.E. Housman. 1991. Isolation, characterization, and expression of the murine Wilms' tumor gene (WT1) during kidney development. *Mol Cell Biol* 11:1707-1712.

217. Armstrong, J.F., K. Pritchard-Jones, W.A. Bickmore, N.D. Hastie, and J.B. Bard. 1993. The expression of the Wilms' tumour gene, WT1, in the developing mammalian embryo. *Mech Dev* 40:85-97.

Chapter II: NO and cGMP upregulate MMP-9 in vascular smooth muscle

This chapter was published in the Journal of Pharmacology and Experimental Therapeutics. Ref: The Journal of pharmacology and experimental therapeutics. 2003 Oct;307(1):429-36. Nitric oxide and cyclic GMP increase the expression of matrix metalloproteinase-9 in vascular smooth muscle. Marcet-Palacios M, Graham K, Cass C, Befus AD, Mayers I, Radomski MW.

1. Title

Nitric Oxide and Cyclic GMP Increase the Expression of Matrix Metalloproteinase-9 in Vascular Smooth Muscle

2. Introduction

Septic shock is characterized by severe hypotension, hyporesponsiveness to vasoconstrictors, and volume depletion leading to multiorgan dysfunction and death (1, 2). This condition is a complex endotoxin-induced systemic inflammatory response, which affects more than 400,000 patients per year in the United States (3). Cell wall endotoxins, such as bacterial lipopolysaccharide (LPS), are largely responsible for the pathogenesis of this disease. The endothelium and the vascular smooth muscle are among major cellular targets of LPS (4).

The deleterious effects of LPS are often mediated by the enzymatic activity of LPS-inducible proteins, such as inducible nitric-oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and matrix metalloproteinase-9 (MMP-9). Initially, these enzymes are

induced in a highly organized fashion to compensate for the damaging effects of LPS, maintain homeostasis, and to contribute to the systemic inflammatory response. However, the overwhelming concentrations of these inducible enzymes can become harmful to the body, contributing to multi organ dysfunction and death. The increased expression of iNOS, for example, results in massive vasodilatation and hypotension (5, 6). Additionally, fast reaction of NO with superoxide (O_2^-) leads to the highly reactive species peroxynitrite ($ONOO^-$) (7, 8). The latter is thought to be responsible for many deleterious effects of NO, acting as an oxidant itself, or leading to the formation of other reactive species (9). Moreover, COX-2 up-regulation leads to overproduction of thromboxane and prostaglandin E_2 (PGE_2), which have been implicated in the pathogenesis of septic shock (10-12). In the presence of LPS, cells like neutrophils secrete considerable amounts of latent MMPs, including MMP-9 (13). Reactive oxygen species produced by the same cell type lead to the chemical activation of MMP-9, and as a consequence, degradation of the vascular wall takes place (14). Other lines of evidence from mice and humans have also correlated MMP-9 levels with shock conditions (13, 15, 16).

Despite solid evidence that iNOS, COX-2, and MMP-9 are involved in the pathogenesis of cellular damage caused by LPS, the interactions (cross talk) between these enzymes are unclear. We found that the simultaneous induction of these enzymes could be triggered by IFN-γ, phorbol 12-myristate13-acetate (PMA), and LPS in rat vascular smooth muscle (VSM) cells, allowing us to study the hypothesis that these three enzymes cross talk to each other. The production of these three enzymes was studied at the transcriptional, protein, and enzyme activity levels. The

results of our experiments provide evidence that iNOS up-regulates COX-2 and MMP-9 gene expression in VSM.

3. Materials and methods

Materials. Polyclonal antibodies against iNOS and COX-2 were obtained from BD Transduction Laboratories (Basingtoke, Hampshire, UK) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Monoclonal antibodies against MMP-9 were obtained from OncogeneScience (Cambridge, MA). RNeasy Mini Kit was obtained from QIAGEN (Mississauga, ON, Canada).

Cell Culture. Rat aortic VSM (A7r5) were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in 75-cm^2 flasks in a humidified atmosphere with 5% CO_2 at 37°C. The medium was Dulbecco's modified eagle's medium supplemented to contain 4.5 g/liter glucose, 1.5 g/liter sodium bicarbonate, 10% fetal bovine serum, gentamycin sulfate (0.05 mg/ml), penicillin G (0.06 mg/ml), and streptomycin sulfate (0.01 mg/ml). Cells were grown until they were confluent and then treated for 12 h with a cocktail containing 10 ng/ml IFN-γ, 1 nM PMA, and 10 μg/ml LPS in 15 ml of 1% fetal bovine serum (activating cocktail). When treating with dexamethasone (Dex), cells were preincubated with this chemical for 1 h before induction with the activating cocktail. Dex concentrations of 1.0, 3.0, and 10 μM were tested. At a concentration of 1 μM, Dex did not confer inhibitory effects as detectable by reverse transcription-polymerase chain reaction (RT-PCR). Because 3 μM Dex was found to be a sufficient amount to inhibit transcription of iNOS, COX-2, and MMP-9, all consecutive experiments were performed using 3 μM Dex. When either N^{ω}-nitro-L-arginine methyl ester (L-NAME; 300 μM), 1*H*-[1,2,4]oxadiazolo[4,3-

a]quinoxalin-1-one (ODQ; 0.05, 0.1, 0.5, 1.0, and 5 μM), or superoxide dismutase (SOD) (0.5, 1, 5, 10, 100 U/ml) were used, these compounds were included in the activating cocktail. Cell harvesting was conducted by scraping cells off plates in the presence of 1 ml of homogenizing buffer (50 mM Tris-HCl, 320 mM sucrose, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, pH 7.4).

Gelatin Zymography. This technique was used to measure pro and active MMP-2 and MMP-9 gelatinolytic activity as previously described (17). After cell extraction, samples were immediately subjected to electrophoresis on 7% SDS-polyacrylamide gel electrophoresis copolymerized with gelatin (2 mg/ml) as the substrate. Independent experiments were performed and run on the same gel. Following electrophoresis, gels were washed in 0.1% Triton X-100 (3x for 20 min). The gels were then incubated for 72 h in the zymography buffer containing 25 mM Tris-Cl, 5 mM $CaCl_2$, 142 mM NaCl, and 0.5 mM Na_3N to determine the activity of secreted enzymes. After incubation, the gels were stained with 0.05% Coomassie Brilliant Blue G-250 in a mixture of methanol/acetic acid/water (2.5:1:6.5) and destained in 4% methanol with 8% acetic acid. The gelatinolytic activities were detected as transparent bands against the background of Coomassie Blue-stained gelatin. Enzyme activity was assayed by densitometry using a ScanJet 3c scanner and SigmaGel measurement software. The pro and active forms of MMP-9 were identified as bands at 92 and 88 kDa, respectively.

Inducible and Constitutive Nitric-Oxide Synthase Activity Assay (Citrulline Assay). Nitric-oxide synthase activity in A7r5 cell homogenate was assessed by

measuring the formation of L-[^{14}C]citrulline from L-[U-^{14}C]arginine as previously described (18). Briefly, samples were homogenized by sonication (VibraCell, Danbury, CT) in 1 ml of ice-cold homogenizing buffer followed by centrifugation at 10,000*g* for 20 min at 4°C. Following centrifugation, 40 µl of supernatant was incubated at 37°C for 20 min in assay buffer (pH 7.4) containing 50 mM KH_2PO_4, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM L-citrulline, 20 µM L-arginine, 1.5 mM dithiothreitol, 1.5 mM NADPH, 10 µM tetrahydrobiopterin, 10 µM FAD, 10 µM FMN, and 0.5 µCi/ml L-[U-^{14}C]arginine. The specificity of L-arginine conversion by NOS to L-citrulline was further confirmed using 1.2 mM N^{ω}-nitro-L-arginine methyl ester, a selective inhibitor of NOS. Additionally, 1.5 mM EGTA, a calcium chelating agent, was used to differentiate between Ca^{2+}-dependent and -independent isoforms of NOS. All enzyme activities were expressed as picomoles of product generated per minute per milligram of protein. The limit of detection of this method was 0.05 pmol/min/mg of protein.

Griess Assay. Formation of NO_2^- was measured as previously described (19). Briefly, 50 µl of supernatant was measured by the Griess reaction. Results were expressed as micromolar concentration per 10^6 cells following incubation for 6, 12, 24, 48, and 72 h. Equal volumes of cell-free supernatant and Griess reagent (1% sulfanilamine, 0.1% *N*-(1-naphyl)-ethylene-diamine dihydrochloride, and 2.5% H_3PO_4) were mixed. $NaNO_2$ was used as a standard. Plates were read on a V_{max} kinetic microplate reader (Molecular Devices Corp., Sunnyvale, CA) at 540 nm.

Live-Cell Fluorescence Determination of Intracellular NO. NO production by A7r5 was assayed using DAF-FM, a cell-permeable NO-sensitive fluorescent dye, as

previously described (20). Cells were incubated for 1 h with 10 μM DAF-FM, prior to visualization.

Cyclooxygenase-2 Enzyme Immunoassay. The activity of COX-2 was measured using a prostaglandin E_2 enzyme immunoassay kit (Amershamn Biosciences Inc., Piscataway, NJ) according to manufacturer's instructions. The amounts of PGE_2 were expressed in picrograms per well of PGE_2 with each well containing a cell concentration of 10^5.

Immunoblot Detection. The cells were harvested and homogenized in the homogenization buffer. The homogenates were subjected to 7% SDS-polyacrylamide gel electrophoresis (17), and proteins were identified using anti-MMP-9 antibodies (0.2 μg/ml; OncogeneScience), anti-COX-2 antibodies (2 μg/ml; Santa Cruz Biotechnology, Inc.), and anti-iNOS antibodies (0.125 μg/ml; BD Transduction Laboratories).

RT-PCR and Quantitative Real-Time PCR. Experiments were performed as previously described (21). Total RNA was isolated using the Qiagen RNeasy kit. The RNA was reverse-transcribed with the use of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) using oligo(dT) as primers. Thereafter, PCR was performed in 20-μl reactions with the primer pairs (25 μM) described in Table 2.1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers were run in all reactions as the internal positive control. The PCR products were amplified for 30 cycles. The selected cycle number was chosen to stop the PCR reaction during its log phase to ensure availability of all reagents. Additional RT-PCR-related information is summarized in Table 2.1.

Quantitative Real-Time PCR. Real-time PCR experiments were performed using the TaqMan quantitative RT-PCR reaction (Applied Biosystems, Foster City, CA) as previously described (22). Briefly, an oligonucleotide probe labeled with a fluorescent tag at the 5'-end and a quenching molecule at the 3'-end is hybridized between two PCR primers at the beginning of the reaction. The 5'-nucleotidase activity of *Taq* polymerase, cleaves the fluorescent dye from the probe during each PCR cycle. The fluorescent signal generated is monitored in real time and is proportional to the amount of starting template in the sample. Real-time PCR products were cloned and sequenced to confirm the identity of the mRNAs. The primer and probe sequences are summarized in Table 2.2.

Reagents. Other PCR related chemicals such as 100-bp DNA ladder, Superscript II, *Taq*DNA polymerase, oligo(dT) primer, dNTP, and RNaseOut ribonuclease were obtained from Invitrogen. PMA, ethidium bromide, interferon-γ, lipopolysaccharide, dexamethasone crystalline, L-NAME (Alexis Corporation, San Diego, CA), N^G-monomethyl-L-arginine, ODQ, and superoxide dismutase were obtained from Sigma Diagnostics, Canada (Mississauga, ON, Canada). Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (MnTMPyP) and 8-bromo-cGMP were obtained from Calbiochem (Mississauga, ON Canada). L-[U-^{14}C]arginine was obtained from Amersham Biosciences (Oakville, ON, Canada) and AG50W-X8 resin was obtained from Bio-Rad (Hercules, CA).

Statistics. Results are means ± S.E. of at least three independent experiments. They were analyzed using one-way analysis of variance, and when significant differences

were found, the multiple comparison Tukey-Kramer test was used (GraphPad InStat). Values where $P < 0.05$ were considered statistically significant.

4. Results

Since the objective of this research was to investigate the interactions between COX-2, iNOS, and MMP-9 in VSM we have first tested various combinations of cytokine/inducing agents to cause simultaneous induction of these enzymes. The following combinations have been tested. A concentration gradient of 0.01, 0.1, 1, and 10 μg/ml LPS did not produce a band detectable by zymography or RT-PCR for MMP-9, but constitutive MMP-2 activity was constant for every treatment (data not shown). PMA (100 nM) produced detectable bands for MMP-9 through RT-PCR and zymography but did not activate iNOS mRNA as detectable by RT-PCR (data not shown). IFN-γ at a concentration of 1 mg/ml did not induce any of the mRNA signals for iNOS, COX-2, or MMP-9 (data not shown). After these preliminary experiments, a combination containing 0.1 ng/ml IFN-γ, 10 μg/ml LPS, and 1 nM PMA was found to be most effective in enzyme induction and all subsequent experiments were performed with cells stimulated with IFN-γ, LPS, and PMA. Initial experiments were done using this cocktail and a time course for MMP-9 activity indicated optimal expression by 12 hr after activation (App. 6).

A time course of NO_2^- formation using the Griess reaction showed that there was a significant increase in nitrite formation after 24 hr (13.9 ± 6.03 μM) and after 48 hr (37.6 ± 1.2 μM) of cell activation. Nitrite formation was not detected in control conditions or when activated cells were cotreated with either L-NAME (300 μM) or

1400W (10 nM) (n = 3). A more sensitive fluorimetric assay using the fluorochrome DAF showed NO production by 12 hr after cell induction (Fig. 2.1).

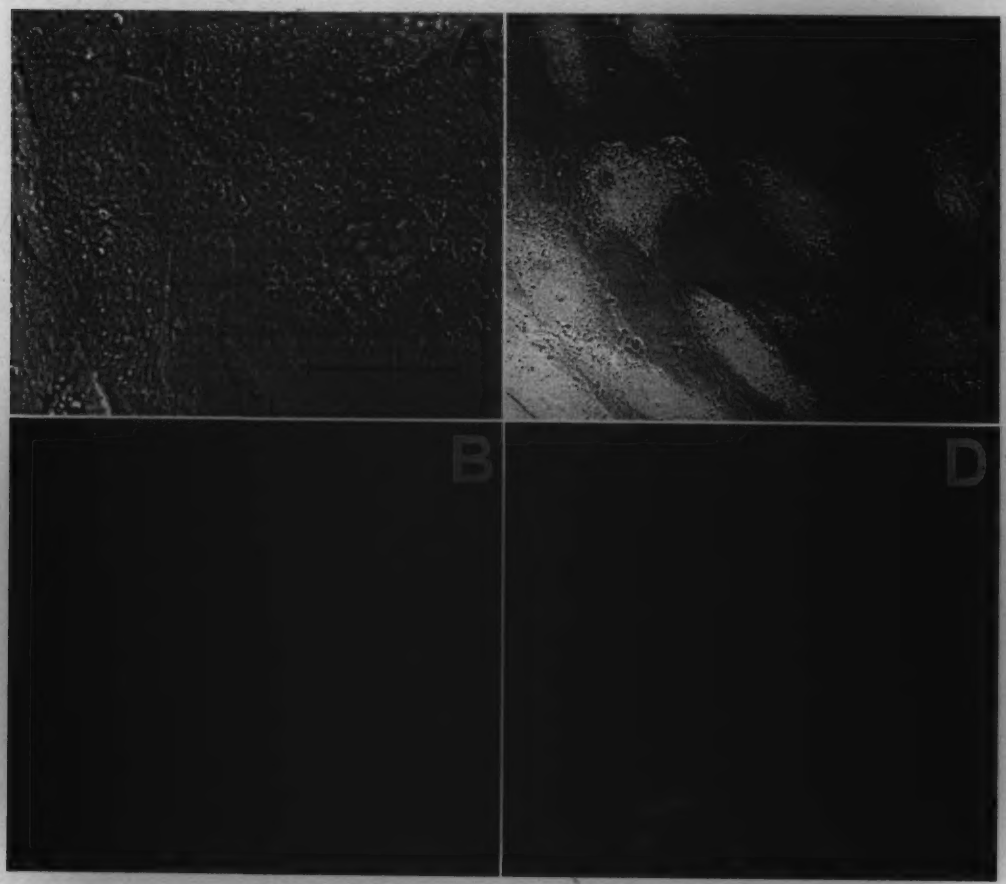


Figure 2.1: Fluorescence detection of intracellular NO production. Cells were either serum-starved (A and B) or treated with a cocktail containing PMA, LPS, and IFN-γ (C and D) for 12 hr then stained with DAF for 1 hr. DAF fluorescence (white in the figure) was analyzed with confocal analysis. Differential interference contrast combined with DAF (B and D) shows cellular morphology (bars = 50 μm).

To study enzyme induction at the transcriptional level, RT-PCR was used. Primers were developed and analyzed using the BLAST sequencing program at GenBank to

ensure unique complementation. When A7r5 cells were exposed to the activating cocktail, the mRNAs for iNOS, COX-2, and MMP-9 were markedly up-regulated (Fig. 2.2, A and B). The PCR products for all templates were identified at their predicted molecular weights of 241, 347, 664, and 528 bp for MMP-9, iNOS, COX-2, and GAPDH, respectively (Fig. 2.2A). The induction of iNOS, COX-2, and MMP-9 mRNAs was significantly inhibited by 3 μM Dex (Fig. 2.2, B and C). The housekeeping gene GAPDH was amplified for each experiment and used as the internal positive control. GAPDH mRNA levels were not significantly changed by different treatments (Fig. 2.2B).

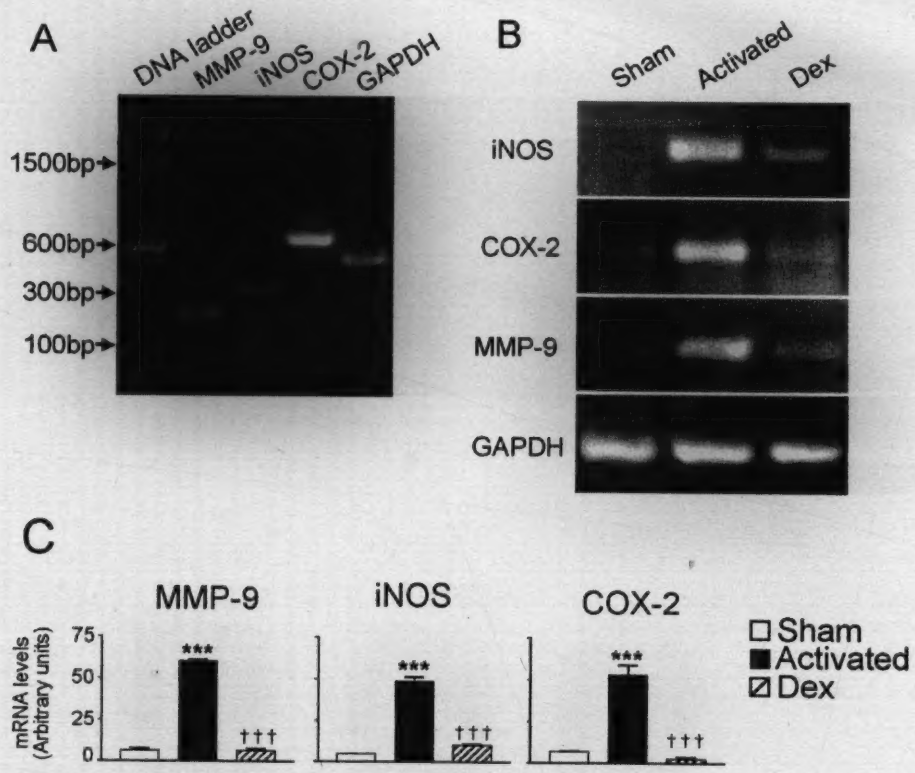


Figure 2.2: Characterization of the septic shock model at the transcriptional level. A, A7r5 cells were treated with a cocktail of pro-inflammatory substances

(under *Materials and Methods*) to induce the production of iNOS, COX-2, and MMP-9. The 100-bp DNA ladder marker provides an estimate of the size of each PCR product ($n = 6$). B, RT-PCR was used to investigate the mRNA levels corresponding to each gene. Cells were untreated (Sham), induced with the activating cocktail (Activated), or incubated with dexamethasone (Dex) 1 hr before activation. GAPDH was used as the positive control for each experiment ($n = 3$). C, densitometric analysis for RT-PCR experiments shown in panel B ($n = 3$). Statistical significance: $P < 0.001$ (***), (†††).

To investigate whether protein levels for these enzymes were affected by the treatment, Western blot experiments were conducted using the same experimental conditions. We found that the levels of iNOS, COX-2, and MMP-9 proteins were increased by the treatment with the activating cocktail, and this enhancement was abolished in the presence of dexamethasone (Fig. 2.3, A–C).

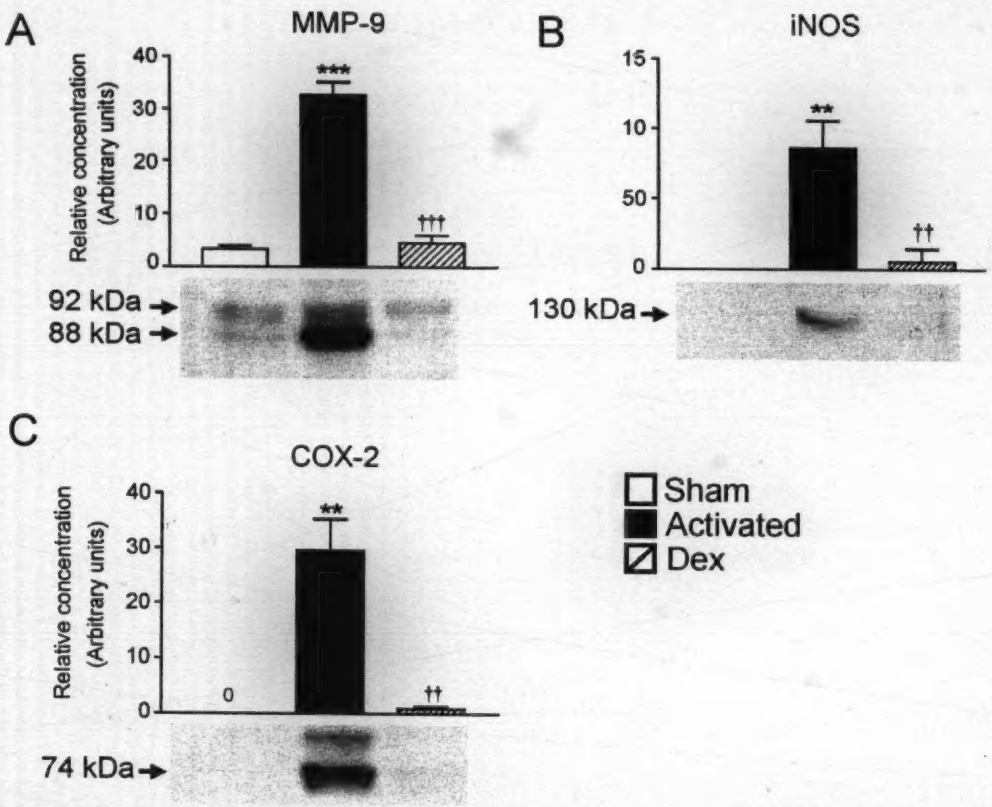


Figure 2.3: Characterization of the septic shock model at the protein level. To further investigate the expression of MMP-9, iNOS, and COX-2, we estimated protein expression levels using immunoblot. A7r5 cells were untreated (Sham), activated with a cocktail (Activated), or pre-incubated with dexamethasone 1 hr before activation. A, the pro and active form of MMP-9 at 92 and 88 kDa were detected. Active MMP-9 was significantly up-regulated in the activated cells. Pretreatment with dexamethasone significantly reduced MMP-9 induction (n = 3). B, iNOS appeared as a band at 130 kDa. Significant differences were found between the sham and activated groups. Also significant differences were obtained when iNOS induction was inhibited with dexamethasone (n = 3). C, similar results to those in panels A and B were obtained for the COX-2 protein at 74 kDa. A second band at a

higher molecular weight also appeared only in activated conditions and was suspected to be due to antibody cross-reactivity ($n = 3$). Statistical significance: $P < 0.01$ (**), (††); $P < 0.001$ (***), (†††).

The enzymatic activities of iNOS, COX-2, and MMP-9 were also characterized. The activity of Ca^{2+}-independent NOS in unstimulated (Sham) VSM was 12.4 ± 0.6 pmol/mg of protein/min (Fig. 2.4A). Cell induction resulted in a significant increase in the activity of this enzyme to 24.4 ± 2.6 pmol/mg of protein/min. Ca^{2+}-dependent NOS activity was not detectable under these conditions, indicating that neither nNOS or eNOS were expressed in A7r5.

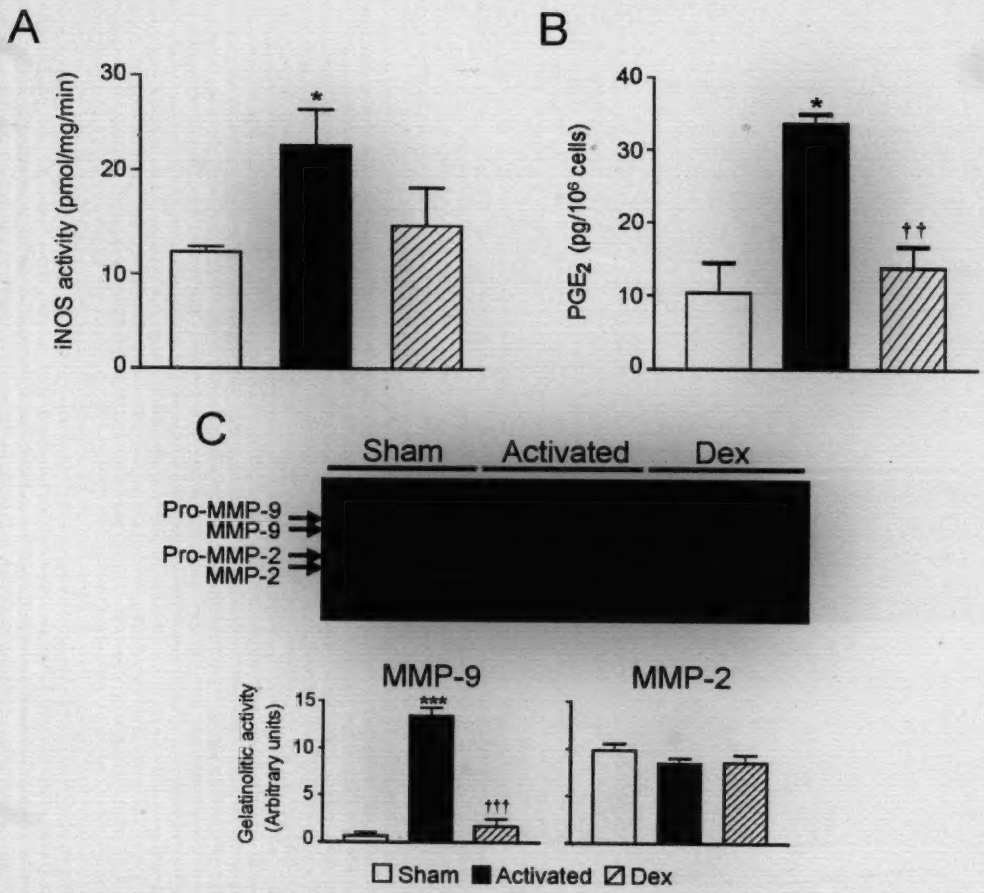


Figure 2.4: MMP-9, iNOS, and COX-2 enzymatic activity in A7r5 cells. A7r5 cells were untreated (Sham), activated with a cocktail (Activated), or pre-incubated with dexamethasone 1 h before activation. A, inducible NOS activity was measured using the citrulline assay. Significant changes between sham and activated conditions were found (P = 0.027), but for iNOS there were no significant changes between sham and Dex treatment. The constitutive NOS activity, also measured with this assay, was not detected after each treatment (n = 3). B, PGE_2 formation was monitored using enzyme-linked immunosorbent assay. Significant changes were observed between sham and activated cells, as well as between activated and

dexamethasone treatment (n = 4). C, zymography was performed to measure the gelatinolytic activity of MMP-9. The pro and active forms of MMP-9 were detected at 92 and 88 kDa, respectively. At 72 and 62 kDa, the pro and active forms of MMP-2 were also detected. MMP-2 is a constitutive metalloproteinase that remained relatively unchanged despite different treatments. Densitometric analysis for MMP-9 and MMP-2 revealed a highly significant up-regulation in activity of MMP-9 in activated cells, whereas Dex inhibited this up-regulation to near control levels (n = 3). Statistical significance: $P < 0.05$ (*), (†); $P < 0.01$ (**), (††); $P < 0.001$ (***), (†††).

The activity of COX-2, as measured by PGE_2 levels, was significantly increased following cell induction (Fig. 2.4B). The gelatinolytic activity of MMP-9, but not MMP-2, was markedly up-regulated by the activating cocktail (Fig. 2.4C). Dexamethasone abolished increases in iNOS, COX-2, and MMP-9 activities caused by cell induction (Fig. 2.4, A-C).

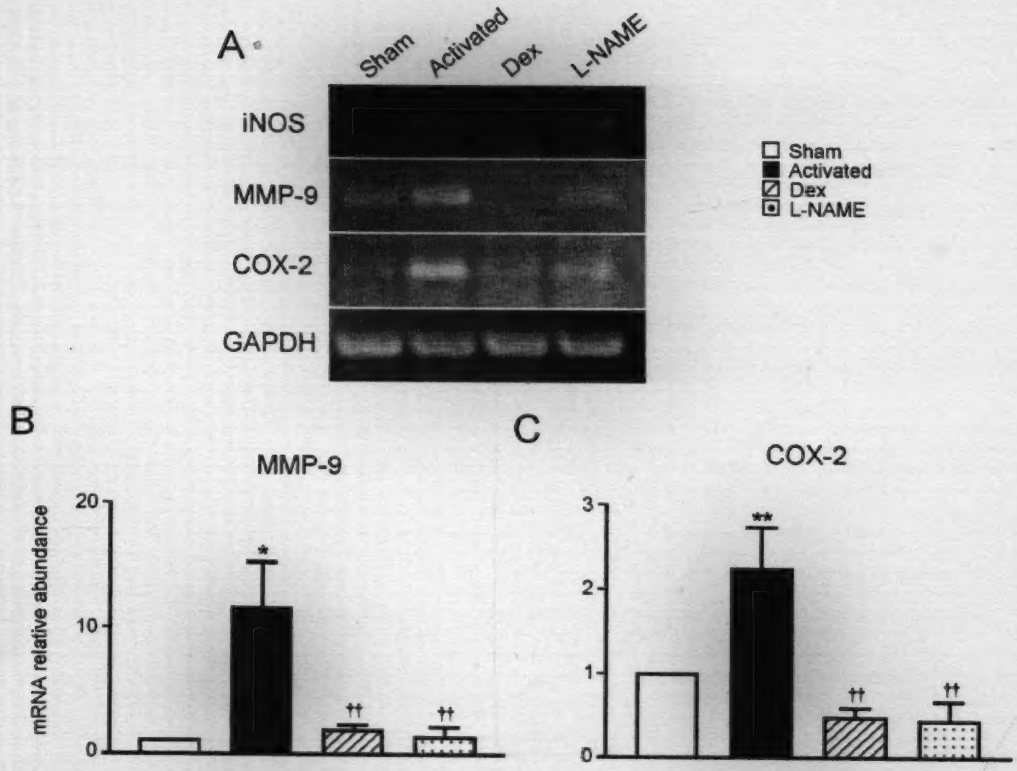


Figure 2.5: Effect of L-NAME treatment on iNOS, COX-2, and MMP-9 mRNA. A, RT-PCR was performed using both Dex and L-NAME as pharmacological tools. The positive control GAPDH was constant for each treatment. L-NAME had an inhibitory effect obvious for COX-2 and for MMP-9 ($n = 3$). B, real-time PCR was performed to validate and quantify the RT-PCR findings. MMP-9 mRNA up-regulation was found to be of 11.7-fold. This effect was inhibited by both dexamethasone and L-NAME to levels relatively close to sham ($n = 3$). C, COX-2 up-regulation during activation was found to be 2.3-fold, and this effect was inhibited to lower than sham levels when treating with either dexamethasone or L-NAME ($n = 3$). Statistical significance: $P < 0.05$ (*), (†); $P < 0.01$ (**), (††).

Effects of L-NAME on COX-2 and MMP-9 Transcription. Inhibition of NOS with L-NAME (300 μM) exerted no significant effect on iNOS mRNA expression in activated cells (Fig. 2.5A). In contrast, COX-2 and MMP-9 transcription was down-regulated by this inhibitor (Fig. 2.5A). L-NAME did not change the expression levels of the positive control GAPDH. To quantify the changes detected using RT-PCR, real-time PCR analysis was conducted for COX-2 and MMP-9 mRNAs. It was found that COX-2 was up-regulated by 2.3-fold (Fig. 2.5C) and that this up-regulation could be inhibited to lower than control levels by either Dex or L-NAME. MMP-9 mRNA levels were up-regulated with the activating cocktail by approximately 11.7 times, and they were significantly reduced in the presence of Dex or L-NAME (Fig. 2.5B).

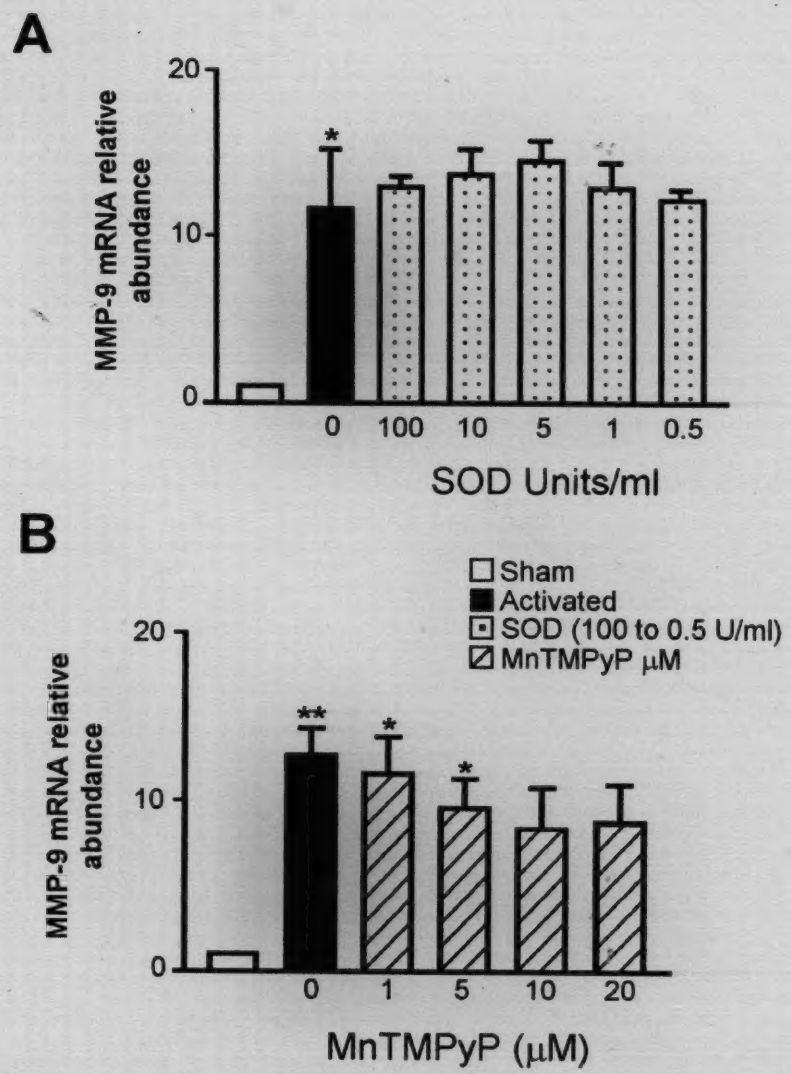


Figure 2.6: MMP-9 gene induction is not mediated by $ONOO^-$. A, an SOD concentration dependence curve was created. Cells were left untreated (Sham) or induced with the activating cocktail containing different concentrations of SOD. No significant variations were observed when comparing maximum activation (0.0 U/ml SOD) with activation in the presence of SOD (0.5–100 U/ml SOD) ($n = 3$). B, the SOD mimetic MnTMPyP (0–20 μM) did not exert a significant reduction of MMP-9 mRNA relative abundance ($n = 3$). Statistical significance: $P < 0.05$ (*).

Effects of SOD and MnTMPyP on MMP-9 mRNA. To investigate whether superoxide and possibly peroxynitrite (23) were involved in MMP-9 regulation by iNOS the scavenger of superoxide, SOD, was tested on A7r5 cells induced with the activating cocktail. SOD at concentrations of 100, 10, 5, 1, and 0.5 U/ml exerted no significant effects on MMP-9 mRNA induction (Fig. 2.6A). These concentrations of SOD effectively blocked superoxide generation by xanthine oxidase (24) (data not shown). The membrane-soluble SOD mimetic MnTMPyP did not modify significantly the up-regulation of these enzymes indicating that neither superoxide nor peroxynitrite are involved in this mechanism (Fig. 2.6B).

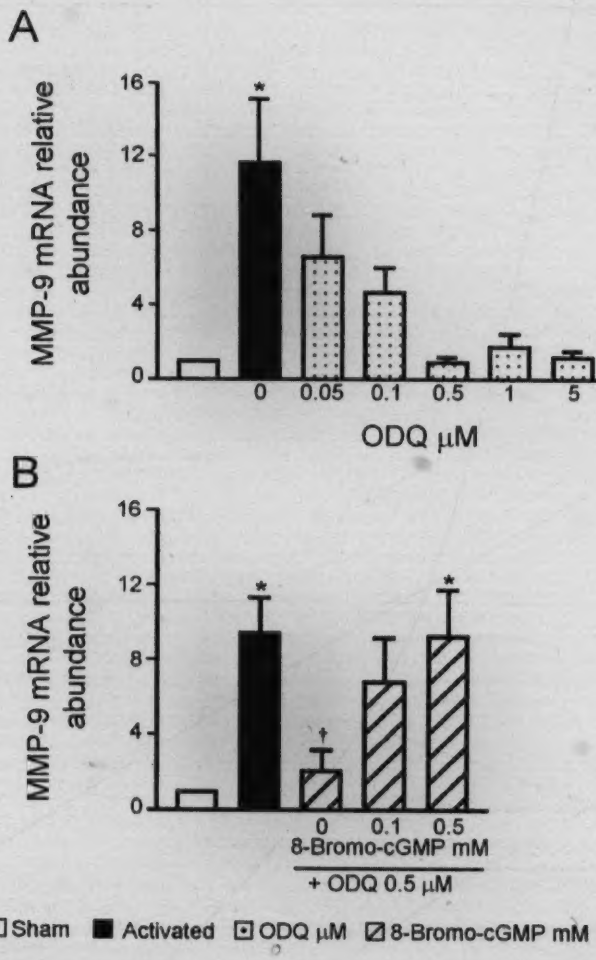


Figure 2.7: Nitric oxide up-regulates MMP-9 via a soluble guanylate cyclase-dependent pathway. Relative abundance of MMP-9 mRNA was measured using real-time PCR. A, there was a significant up-regulation of the MMP-9 gene when inducing A7r5 cells with the activating cocktail (Activated). When cells were activated in the presence of ODQ (0.05–5 μM), this resulted in a significant decrease in the MMP-9 mRNA levels. It was shown that at a concentration of 0.5 μM ODQ the activating cocktail did not induce any significant changes in the MMP-9 mRNA levels ($n = 3$). B, exogenously added 8-bromo-cGMP (0–0.5 mM) reversed the effect of ODQ (0.5 μM). Statistical significance: $P < 0.05$ (*), (†).

Effects of ODQ and 8-bromo-cGMP on MMP-9 mRNA Regulation. To study the involvement of cGMP in MMP-9 mRNA regulation, ODQ, an inhibitor of soluble guanylate cyclase was used. ODQ effectively decreased, in a concentration-dependent fashion, the levels of MMP-9 mRNA (Fig. 2.7A). 8-bromo-cGMP, a soluble analog of cGMP, reversed the effect of ODQ (Fig. 2.7B).

5. Discussion

We have investigated the interactions between iNOS, COX-2, and MMP-9, three pro-inflammatory enzymes that are induced in the vasculature during acute inflammatory reactions such as septicemia and septic shock (5-12, 14, 15). We have studied these interactions using rat smooth muscle vascular cells, which were induced by LPS, IFN-γ, and PMA. Under the experimental conditions used, the inducing agents resulted in up-regulation of expression (mRNA and protein), and activity (product generation or substrate degradation) levels. We have further validated our model of simultaneous induction of iNOS, COX-2, and MMP-9 using dexamethasone, a potent glucocorticoid known to inhibit many pathways of inflammation. As expected, this drug treatment efficiently reduced the expression of iNOS, COX-2, and MMP-9 (Figs. 2.2B and 2.3, A–C).

We then used RT-PCR to study the effects of blockade of NOS with L-NAME on MMP-9 and COX-2 mRNA levels. Inhibition of NOS with L-NAME down-regulated the expression of COX-2. This is not surprising since NO is known to stimulate the activity of cyclooxygenase (25) and NO stimulates COX-2 mRNA expression in rat mesangial cells (26). Interestingly, we have found that MMP-9 mRNA up-regulation was inhibited by L-NAME showing that MMP-9 induction is NO-dependent by a

NOS-dependent mechanism. This observation was confirmed and quantified by real-time PCR experiments (Fig. 2.5A).

Initially it was thought that NO effects could potentially be mediated through either nitrosylation, oxidation, nitration, or a combination of these reactions, since these nonspecific reactions are common in inflammatory settings. Peroxynitrite is one of the most reactive biochemical fates of NO and in many studies has been described as a signaling molecule that acts through tyrosine nitration (23). For this reason, SOD and membrane-soluble SOD mimetic MnTMPyP were used to investigate $ONOO^-$ effects on the MMP-9 induction pathway. The treatments did not modify the MMP-9 gene control mechanism suggesting that $ONOO^-$ was not involved in this process (Fig. 2.6, A and B). A second pathway by which NO could be acting was through soluble guanylate cyclase. Our results indicated that in the presence of ODQ, an inhibitor of soluble guanylate cyclase, the MMP-9 mRNA returned to its basal levels. Furthermore, the effect produced by ODQ was reversed by exogenous 8-bromo-cGMP, a soluble analog of cGMP (Fig. 2.7B). These data imply that the soluble guanylate cyclase was the main pathway for NO-dependent MMP-9 gene up-regulation.

Recently, in contrast to our results on enhancement of MMP-9, Eberhardt et al. (27) using rat mesangial cells found that endogenous or exogenously provided NO decreased MMP-9 gene expression. They also showed that administration of another inhibitor of NOS, N^G-monomethyl-L-arginine increased MMP-9 mRNA expression. NO can reduce $t_{1/2}$ life of MMP-9 mRNA (28) as well as *S*-nitrosylate nuclear factor-κ

B (29), two mechanisms by which NO may modulate MMP-9 expression. By contrast NO has been found to increase MMP-9 activity through *S*-nitrosylation (30).

Interestingly, pharmacological studies using L-NAME (inhibitor of NOS) and AMD6221 [ruthenium(III) polyaminocarboxylate complex; NO scavenger] yielded contradictory data on MMP-9 activity. The administration of L-NAME to neonatal hyperoxic rats increased activity of MMP-9 (17), whereas NO scavenging decreased MMP-9 activity elevated by extracorporeal circulation (31). These complex effects of inhibition by NO of expression and activity of MMP-9 likely reflect the dual nature of NO as inflammatory-inhibitor and inflammatory-mediator (32).

The conflicting results on enhancement or inhibition of MMP-9 illustrate the complexity of the interactions of NO with cell-signaling cascades. Factors such as cell type, stimulation time, and stimulatory cocktail likely contribute to some of the differences observed. In our study, we examined the expression of MMP-9 mRNA after 12 hr of treatment using a cocktail containing LPS, IFN-γ, and PMA and observed that serum-starved cells even in the absence of the stimulatory cocktail (control conditions) began to synthesize MMP-9 mRNA by 24 hr. These results were further validated by a time course of MMP-9 activity, using zymography, in which 12 hr was clearly the optimal induction time as described above. It is possible that in settings where cells have been stimulated for 24 to 72 hr, as done in several of the studies outlined above (27, 28), the levels of NO are different than those we have observed. A time course of NO_2^- formation using the Griess reaction showed that there was a significant increase in nitrite formation after 24 and 48 h of cell activation,

whereas nitrite formation could not be detected in control conditions or when activated cells were cotreated with either L-NAME or 1400W.

Thus, NO may function through alternative pathways to reduce MMP-9 levels, e.g., through oxidation of MMP-9 mRNA. Our findings could be interpreted in terms of early and late NO-mediated events, in which NO concentration and exposure time are critical in dictating outcomes. Future experiments must attempt to clarify the underlying causes of the contradictory results in the literature.

The list of genes that are up-regulated via soluble guanylate cyclase-mediated pathways includes only COX-2, tumor necrosis factor, PAI-1, FLT-1, MKP-1 (33), and now MMP-9. The exact mechanism by which cGMP exerts its transcription regulatory functions has not been fully elucidated. Activated soluble guanylate cyclase synthesizes cGMP, which in turn alters the activity of three main target proteins: 1) cGMP-regulated ion channels, 2) cGMP-regulated phosphodiesterases, and 3) cGMP-dependent protein kinases (PKG). Several lines of evidence appear to implicate PKG as the mediator of soluble guanylate cyclase action on the MMP-9 gene (34-36). More recent studies have shown that PKG-mediated gene regulation takes place via activation of members of the mitogen-activated protein kinase protein family including Raf1, extracellular signal-regulated kinase 1/2, and synthesis of c-Jun. Interestingly, protein kinase C (PKC) has been shown to regulate transcription of MMP-9 gene via stimulation of extracellular signal-regulated kinase 1/2 pathway (37, 38). This PKC-like behaviour may partially explain how increased soluble guanylate cyclase activity up-regulates MMP-9 gene. However, under the conditions of our experiments, stimulation of PKG but not PKC appears to be necessary for MMP-9

gene induction since, despite the continuous presence of PMA a known stimulator of PKC (39), the blockade of soluble guanylate cyclase pathway with ODQ abolished MMP-9 gene transcription.

The precise mechanisms involved in soluble guanylate cyclase-mediated MMP-9 gene induction remain to be studied. It is important to note that our current understanding of regulatory mechanisms of the MMP-9 promoter is very limited. Transcription Elements Search System (TESS) analysis of 1,300 bp of the rat MMP-9 promoter reveals a complex picture of multiple binding sites for more than 200 different transcription factors. We are currently investigating the relevance of a novel PKG/protein kinase A target, which we have identified as a possible MMP-9 repressor.

The results of our experiments show that the NO-cGMP pathway plays a crucial role in MMP-9 gene regulation. The pharmacological significance of our findings remains to be studied.

6. Tables

Table 2.1: Summary of RT-PCR primer details.

mRNA	Primer Position (5' to 3')	PCR Product Size	Primer Sequence (5' to 3')	Annealing Temperature	Accession Number
		bp		°C	
F-MMP-9	141-164	241	gga tta cct gta ccg cta tgg tta	55	NM_031055
R-MMP-9	381-358		ttg gat cca ata ggt gat gtt atg		
F-COX-2	331-354	664	cac ccc aaa cac agt aca cta cat	58	S67722
R-COX-2	994-971		gat ggt agc ata cat cat cag acc		
F-iNOS	227-250	347	caa gct gta tgt gac tcc atc gac	55	D14051
R-iNOS	573-549		aga tga gct cat cca gag tga gct g		
F-GAPDH	331-350	528	acc acc atg gag aag gct gg	55	NM_017008
R-GAPDH	858-839		ctc agt gta gcc cag gat gc		

Table 2.2: Summary of real time PCR primer and probe sequences and accession numbers

mRNA	Primer Sequence (5' to 3')	Probe Sequence (5' to 3')	Accession Number
F-MMP-9	cag acc aag ggt aca gcc tgt t	6FAM-5'-ctg gtg gca gcg ca	NM_031055
R-MMP-9	agc gca tgg ccg aac tc		
F-COX-2	tcc agc tca cgg tac cag aag	6FAM-5'-tag gcc cca tgc aat	S67722
R-COX-2	gca tcc agg ctg aac tca ca		
F-iNOS	gga ttt tcc cag gca acc a	6FAM-5'-acg gcc ctg gtg ca	D14051
R-iNOS	tcc aca act cgc tcc aag atc		
F-GAPDH	cct gga gaa acc tgc caa gta t	6FAM-5'-atg aca tca aga agg tgg	NM_017008
R-GAPDH	ctc ggc cgc ctg ctt		

7. References

1. Bone, R.C. 1994. Gram-positive organisms and sepsis. *Arch Intern Med* 154:26-34.

2. Webber, D. 1998. Restored credibility for sepsis therapeutics? *Nat Biotechnol* 16:1303.

3. Knuefermann, P., S. Nemoto, G. Baumgarten, A. Misra, N. Sivasubramanian, B.A. Carabello, and J.G. Vallejo. 2002. Cardiac inflammation and innate immunity in septic shock: is there a role for toll-like receptors? *Chest* 121:1329-1336.

4. Alexander, C., and E.T. Rietschel. 2001. Bacterial lipopolysaccharides and innate immunity. *J Endotoxin Res* 7:167-202.

5. Gomez-Jimenez, J., A. Salgado, M. Mourelle, M.C. Martin, R.M. Segura, R. Peracaula, and S. Moncada. 1995. L-arginine: nitric oxide pathway in endotoxemia and human septic shock. *Crit Care Med* 23:253-258.

6. Titheradge, M.A. 1999. Nitric oxide in septic shock. *Biochim Biophys Acta* 1411:437-455.

7. Beckman, J.S., T.W. Beckman, J. Chen, P.A. Marshall, and B.A. Freeman. 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* 87:1620-1624.

8. Beckman, J.S., and W.H. Koppenol. 1996. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 271:C1424-1437.

9. Szabo, C., B. Zingarelli, and A.L. Salzman. 1996. Role of poly-ADP ribosyltransferase activation in the vascular contractile and energetic failure elicited by exogenous and endogenous nitric oxide and peroxynitrite. *Circ Res* 78:1051-1063.

10. Ermert, M., M. Merkle, R. Mootz, F. Grimminger, W. Seeger, and L. Ermert. 2000. Endotoxin priming of the cyclooxygenase-2-thromboxane axis in isolated rat lungs. *Am J Physiol Lung Cell Mol Physiol* 278:L1195-1203.

11. Fischer, L.G., M.W. Hollmann, D.J. Horstman, and G.F. Rich. 2000. Cyclooxygenase inhibitors attenuate bradykinin-induced vasoconstriction in septic isolated rat lungs. *Anesth Analg* 90:625-631.

12. Strong, V.E., P.J. Mackrell, E.M. Concannon, H.A. Naama, P.A. Schaefer, G.W. Shaftan, P.P. Stapleton, and J.M. Daly. 2000. Blocking prostaglandin E2 after trauma attenuates pro-inflammatory cytokines and improves survival. *Shock* 14:374-379.

13. Albert, J., A. Radomski, A. Soop, A. Sollevi, C. Frostell, and M.W. Radomski. 2003. Differential release of matrix metalloproteinase-9 and nitric oxide following infusion of endotoxin to human volunteers. *Acta Anaesthesiol Scand* 47:407-410.

14. Opdenakker, G. 2001. New insights in the regulation of leukocytosis and the role played by leukocytes in septic shock. *Verh K Acad Geneeskd Belg* 63:531-538; discussion 538-541.

15. Pugin, J., M.C. Widmer, S. Kossodo, C.M. Liang, H.L.n. Preas, and A.F. Suffredini. 1999. Human neutrophils secrete gelatinase B in vitro and in vivo

in response to endotoxin and proinflammatory mediators. *Am J Respir Cell Mol Biol* 20:458-464.

16. Dubois, B., S. Starckx, A. Pagenstecher, J. Oord, B. Arnold, and G. Opdenakker. 2002. Gelatinase B deficiency protects against endotoxin shock. *Eur J Immunol* 32:2163-2171.

17. Radomski, A., G. Sawicki, D.M. Olson, and M.W. Radomski. 1998. The role of nitric oxide and metalloproteinases in the pathogenesis of hyperoxia-induced lung injury in newborn rats. *Br J Pharmacol* 125:1455-1462.

18. Radomski, M.W., P. Vallance, G. Whitley, N. Foxwell, and S. Moncada. 1993. Platelet adhesion to human vascular endothelium is modulated by constitutive and cytokine induced nitric oxide. *Cardiovasc Res* 27:1380-1382.

19. Gilchrist, M., M. Savoie, O. Nohara, F.L. Wills, J.L. Wallace, and A.D. Befus. 2002. Nitric oxide synthase and nitric oxide production in in vivo-derived mast cells. *J Leukoc Biol* 71:618-624.

20. Grisham, M.B., D. Jourd'Heuil, and D.A. Wink. 1999. Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites:implications in inflammation. *Am J Physiol* 276:G315-321.

21. Szkotak, A.J., A.M. Ng, J. Sawicka, S.A. Baldwin, S.F. Man, C.E. Cass, J.D. Young, and M. Duszyk. 2001. Regulation of K(+) current in human airway epithelial cells by exogenous and autocrine adenosine. *Am J Physiol Cell Physiol* 281:C1991-2002.

22. Ritzel, M.W., A.M. Ng, S.Y. Yao, K. Graham, S.K. Loewen, K.M. Smith, R.G. Ritzel, D.A. Mowles, P. Carpenter, X.Z. Chen, E. Karpinski, R.J. Hyde,

S.A. Baldwin, C.E. Cass, and J.D. Young. 2001. Molecular identification and characterization of novel human and mouse concentrative Na+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). *J Biol Chem* 276:2914-2927.

23. Patel, R.P., J. McAndrew, H. Sellak, C.R. White, H. Jo, B.A. Freeman, and V.M. Darley-Usmar. 1999. Biological aspects of reactive nitrogen species. *Biochim Biophys Acta* 1411:385-400.

24. Ohara, Y., T.E. Peterson, and D.G. Harrison. 1993. Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest* 91:2546-2551.

25. Salvemini, D., T.P. Misko, J.L. Masferrer, K. Seibert, M.G. Currie, and P. Needleman. 1993. Nitric oxide activates cyclooxygenase enzymes. *Proc Natl Acad Sci U S A* 90:7240-7244.

26. Tetsuka, T., D. Daphna-Iken, B.W. Miller, Z. Guan, L.D. Baier, and A.R. Morrison. 1996. Nitric oxide amplifies interleukin 1-induced cyclooxygenase-2 expression in rat mesangial cells. *J Clin Invest* 97:2051-2056.

27. Eberhardt, W., T. Beeg, K.F. Beck, S. Walpen, S. Gauer, H. Bohles, and J. Pfeilschifter. 2000. Nitric oxide modulates expression of matrix metalloproteinase-9 in rat mesangial cells. *Kidney Int* 57:59-69.

28. Eberhardt, W., S. Akool el, J. Rebhan, S. Frank, K.F. Beck, R. Franzen, F.M. Hamada, and J. Pfeilschifter. 2002. Inhibition of cytokine-induced matrix metalloproteinase 9 expression by peroxisome proliferator-activated receptor alpha agonists is indirect and due to a NO-mediated reduction of mRNA stability. *J Biol Chem* 277:33518-33528.

29. Marshall, H.E., and J.S. Stamler. 2001. Inhibition of NF-kappa B by S-nitrosylation. *Biochemistry* 40:1688-1693.

30. Gu, Z., M. Kaul, B. Yan, S.J. Kridel, J. Cui, A. Strongin, J.W. Smith, R.C. Liddington, and S.A. Lipton. 2002. S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science* 297:1186-1190.

31. Mayers, I., T. Hurst, A. Radomski, D. Johnson, S. Fricker, G. Bridger, B. Cameron, M. Darkes, and M.W. Radomski. 2003. Increased matrix metalloproteinase activity after canine cardiopulmonary bypass is suppressed by a nitric oxide scavenger. *J Thorac Cardiovasc Surg* 125:661-668.

32. Droge, W. 2002. Free radicals in the physiological control of cell function. *Physiol Rev* 82:47-95.

33. Pfeilschifter, J., W. Eberhardt, and K.F. Beck. 2001. Regulation of gene expression by nitric oxide. *Pflugers Arch* 442:479-486.

34. Gudi, T., G.K. Hong, A.B. Vaandrager, S.M. Lohmann, and R.B. Pilz. 1999. Nitric oxide and cGMP regulate gene expression in neuronal and glial cells by activating type II cGMP-dependent protein kinase. *Faseb J* 13:2143-2152.

35. Gudi, T., I. Huvar, M. Meinecke, S.M. Lohmann, G.R. Boss, and R.B. Pilz. 1996. Regulation of gene expression by cGMP-dependent protein kinase. Transactivation of the c-fos promoter. *J Biol Chem* 271:4597-4600.

36. Gudi, T., S.M. Lohmann, and R.B. Pilz. 1997. Regulation of gene expression by cyclic GMP-dependent protein kinase requires nuclear translocation of the

kinase: identification of a nuclear localization signal. *Mol Cell Biol* 17:5244-5254.

37. Genersch, E., K. Hayess, Y. Neuenfeld, and H. Haller. 2000. Sustained ERK phosphorylation is necessary but not sufficient for MMP-9 regulation in endothelial cells: involvement of Ras-dependent and -independent pathways. *J Cell Sci* 113 Pt 23:4319-4330.

38. Lee, W.J., C.Y. Shin, B.K. Yoo, J.R. Ryu, E.Y. Choi, J.H. Cheong, J.H. Ryu, and K.H. Ko. 2003. Induction of matrix metalloproteinase-9 (MMP-9) in lipopolysaccharide-stimulated primary astrocytes is mediated by extracellular signal-regulated protein kinase 1/2 (Erk1/2). *Glia* 41:15-24.

39. Ron, D., and M.G. Kazanietz. 1999. New insights into the regulation of protein kinase C and novel phorbol ester receptors. *Faseb J* 13:1658-1676.

Chapter III: WT1 regulates MMP-9 via nitric oxide

This chapter has been submitted for publication

1. Title

The Transcription Factor Wilms Tumor 1 Regulates Matrix Metalloproteinase-9 through a Nitric Oxide-Mediated Pathway.

2. Introduction

Matrix metalloproteinases (MMP) degrade collagens and other extracellular matrix proteins and are important to normal turnover or remodeling of extracellular matrix proteins (1). MMP also degrade other proteinases as well as clotting factors, receptors and cell adhesion proteins (2), thereby implicating this family in a multitude of homeostatic and pathogenic processes.

Matrix metalloproteinase-9 (MMP-9), also called gelatinase-B, is a type IV collagenase present in low quantities in the healthy lung, while increased expression has been observed in several lung diseases including idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease (COPD) and asthma (1). Indeed, MMP-9 expression in the lungs correlates with lung injury as well as with epithelial cell death (3). Furthermore, individuals with allergic asthma have elevated levels of MMP-9,

both in the lung and the blood (4-6) and airway allergen challenge is associated with increased MMP-9 levels (6). MMP-9 knockout mice have heightened airway hyperresponsiveness to methacholine and expression of Th2 cytokines in response to ovalbumin-induced lung inflammation (7). Several cell types within the lung express MMP-9, including structural and inflammatory cells (1) and *in vitro* studies indicate that it is produced by lung epithelial cells (LEC) stimulated with TNF and IFN-γ or by bacterial products such as lipopolysaccharide (LPS) (8, 9). MMP-9 plays a role in dendritic cell recruitment during allergen-induced airway inflammation (10, 11) and its deficiency impairs cellular infiltration and bronchial hyperresponsiveness (12). Therefore, in addition to remodeling, MMP-9 may also be important in development of adaptive immune responses in the lung.

Nitric oxide (NO) is an important regulator of MMP-9 (13-17) and we have previously shown this appears to be through a soluble guanylate cyclase (sGC)-dependent pathway (18). Moreover, mice with mutant iNOS produce significantly less MMP-9 than wild type mice (19, 20). Despite an established role for MMP-9 in asthma and studies demonstrating pro-inflammatory stimuli such as cytokines, LPS and NO inducing its expression, transcriptional regulation of MMP-9 is still not well characterized.

In an effort to identify potential regulatory elements of MMP-9, we performed comparative genomic analysis. The most highly conserved non-coding sequence in more than 10kb spanning the MMP-9 gene and flanking region was within the 300 base pairs 5' of the transcription initiation site. This region contains a CA repetitive element that is reported to be polymorphic, ranging from 14-28 repeats within a

Caucasian population (21). Furthermore, one study indicates that MMP-9 transcription may be inversely associated with the length of the CA repeat (22). *In silico* analysis of the conserved MMP-9 promoter region indicated the CA repeat binds a transcription factor called Wilms Tumor 1 (WT1).

Although expression of WT1 has been primarily considered in relation to development of Wilms tumor, this transcription factor is expressed by a number of tissues during development as well as throughout life, suggesting it also plays a role in tissue homeostasis (23). While, there are no reports of WT1 expression in lung epithelium, both kidney epithelial and breast epithelial cells have been reported to express this factor (24). Interestingly, WT1 is regulated by PKA (24-26) a downstream mediator of sGC (27-29). PKA-mediated phosphorylation of WT1 induces its translocation from the nucleus to the cytosol. Since WT1 has been shown to be a transcriptional repressor (30-32), PKA phosphorylation may result in over-expression of target genes.

Our findings indicate that in human LEC WT1 is a transcriptional repressor of MMP-9, mediated through a NO/sGC/PKA-dependent pathway(s). Further study is now needed to determine whether clinical conditions exhibiting tissue remodeling, such as asthma and/or COPD, demonstrate reduced levels or activities of WT-1.

3. Materials and methods

Cell culture: Primary normal human bronchial epithelial cells (**PBEC**) were obtained from Clonetics (Clonetics Corporation, Walkerville, MD) and grown in bronchial EC growth medium supplied by Clonetics. The human bronchial epithelial cell line **HS-24** was generously provided by Dr. E. Spiess (Heidelberg, Germany) and cultured

using RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 2 mM L-glutamine (Sigma, Oakville, ON, Canada). **BEAS-2b**, human bronchial epithelial cells transformed by adenovirus 12-SV40 were obtained from American Type Collection (ATCC) and cultured using BEGM Bullet Kit obtained from Clonetics with no additional fetal bovine serum (FBS). The human lung adenocarcinoma cell line **Calu-3** was obtained from ATCC and cultured with minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. The human lung carcinoma epithelial cell line **A549** was obtained from ATCC and cultured in Ham's F12K supplemented with 2 mM L-glutamine. All of the above cells were cultured at 37°C, 5% CO_2 and 10% FBS (Hyclone, Logan, UT) unless specified.

Materials: N{omega}-nitro-L-arginine methyl ester (L-NAME) was obtained from Alexis Corporation (San Diego, CA). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was obtained from Sigma-Aldrich (Mississauga, ON, Canada), Myr-GRTGRRNAI-NH_2 (Myr-PKA) a PKA inhibitor and DT-3, a PKG inhibitor and forskolin were obtain from Calbiochem. (San Diego, CA)

Gelatin zymography: This technique was performed as previously described with some modifications (18). MMP-9 levels in A549 were low and could not be detected in lysates. To detect MMP-9, media was collected (10 ml) after 12 hr of treatment and concentrated using Centricon centrifugal filters (Millipore, Billerica, MA) down to 200 µl. Earlier time points were tested using concentrated media (1, 3 and 6 hr) but the MMP-9 signal was undetectable or too low for densitometric analysis. Samples were subjected to electrophoresis on 10% zymography gels (Bio-Rad Laboratories,

Mississauga, Ontario). Following electrophoresis, gels were washed in 0.1% Triton X-100 (3 times for 20 min). The gels were then incubated for 72 hr in the zymography buffer containing 25 mM Tris-Cl, 5 mM CaCl2, 142 mM NaCl, and 0.5 mM Na3N to determine the activity of secreted enzymes. After incubation, the gels were stained with 0.05% Coomassie Brilliant Blue G-250 in a mixture of methanol/acetic acid/water (2.5:1:6.5) and destained in 4% methanol with 8% acetic acid. The gelatinolytic activities were detected as transparent bands against the background of Coomassie Blue-stained gelatin. Enzyme activity was assayed by densitometry using the Odyssey Imaging System (Li-cor Biosciences, Nebraska, USA) and SigmaGel obtained from Sigma-Aldrich (Mississauga, ON, Canada).

Reverse Transcription Polymerase Chain Reaction (RT-PCR): RT-PCR was performed as previously described (18). WT1 primers were designed to span from exon 4 to exon 6, thus detecting the presence or absence of exon 5, a known spliced exon that results in WT1 variants. The primer sequences used were developed based on the WT1 sequence of accession number NM_024426. The forward primer sequence was 5' GAT GAA CTT AGG AGC CAC CTT AAA 3' and the reverse primer sequence was 5' TAT GTC TCC TTT GGT GTC TTT TGA 3', which generated 412 and 361 bp long PCR products. MMP-9 primers were designed from the sequence of accession number NM_004994.2. The forward primer was 5' CCT GCC AGT TTC CAT TCA TC 3' and reverse 5' CTA CGG CCA CTA CTG TGC CT 3' amplified a 665bp PCR product. Beta-actin (β-actin) was used as the internal positive control. β-actin primers were forward 5' GGCATC CTC ACC CTG AAG TA 3' and reverse 5' AGG GCA TAC CCC TCG TAG AT 3' generated form the

sequence NM_001101. These primers amplified a 325 bp PCR product. Inducible NOS (iNOS) primers were forward 5' CAC TGA GCT CAT CCC CTT CT 3' and reverse 5' TGT GCT CTT TGC CTG TAT GC 3' using accession number NM_153292.1 generated a 428 bp PCR product. Primers were analyzed using the BLAST sequencing program at Genbank to ensure unique complementation of *Homo sapiens*. PCR amplification was performed in cycles of 1 min at 94°C, 1 min at 60 C, and 2 min at 72°C, and a final cycle of 72°C for 10 min to complete polymerization. The number of cycles was optimized to be in the exponential phase of the reaction by performing the reaction at different cycles. Densitometric analysis of the gels was performed to select optimal PCR cycle numbers. MMP-9 and iNOS were run for 29 cycles, β-actin for 25, whereas WT1 was run for 30. PCR products were analyzed on a 2% agarose gel containing ethidium bromide.

Cloning and sequencing of PCR products: The amplified PCR products were cloned into pCR2.1 plasmid vector using the T/A cloning kit (Invitrogen Life Technologies, Burlington, ON, Canada) as previously described (33). Double-stranded DNA sequencing was conducted using an ABI 373A automated sequencer (Applied Biosystems, Foster City, CA) and sequences were tested for homology using the BLAST sequencing program at Genbank.

Comparative genomic analysis: Assessment of sequence conservation of MMP-9 across multiple species was performed using the VISTA program (Mayor, C 2000 Bioinformatics) located at www-gsd.lbl.gov/vista/.

Antibodies: Monoclonal antibodies against WT1 were obtained from Santa Cruz Biotechnologies, Inc. (F-6) (Santa Cruz, CA) and from Oncogene (Ab-1) (Oncogene

Science, San Diego, CA). WT1 polyclonal antibody (N20) and its peptide blocker (N20 P) were obtained from Santa Cruz Biotechnology. Antibodies used in ChiP, other than N20, were normal rabbit IgG and anti—acetyl-Histone H3 (Upstate Cell Signaling Solutions, VA, USA). Other antibodies included: peroxidase-conjugated goat anti-mouse IgG (BD biosciences. San Diego, CA), donkey anti-mouse IgG, IRDye 800 conjugated (Rockland Immunochemicals, Inc, Gilbertsville, PA), mouse IgG1 isotype control (R&D Systems, Minneapolis), peroxidase-conjugated affinity purified F(ab')2 fragment goat anti-mouse IgG (H + L) (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) and Rhodamine Red-X goat anti-mouse IgG.

Immunoprecipitation: Following stimulation (see below), cells were harvested (10^7 cells) and washed twice in cold PBS and spun at 400g for 10 min. Cells were then resuspended in lysis buffer (10 mM Hepes, 2 mM $MgCl_2$, 15 mM KCl, 0.1 mM EDTA, and 0.15% Nonidet P-40, containing a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) and precleared with 50 µl of Protein A/G beads (Pierce Biotechnology, Inc, Rockford, IL). Ten µg of antibody were added to lysate and incubated for 1 hr. Then 50 µl of Protein A/G beads were incubated for 1 hr at 4°C. Collected beads were then used for Western blot analysis or 2-dimensional electrophoresis (2-D electrophoresis). Cells were stimulated for 10 min with a cocktail containing 10 µg/ml bacterial lipopolysaccharide (LPS), 1 ng/ml interferon-gamma (IFN-γ), and 1 nM phorbol 12-myristate13-acetate (PMA) or with only 10 ng/ml human recombinant tumor necrosis factor (TNF) (Sigma Oakville, ON, Canada).

Isolation of WT1 using its DNA binding site: Streptavidin coated paramagnetic particles (PMP) (Promega Corporate, Madison, WI) were incubated for 3 hr with a dsDNA oligo, corresponding to the WT1 binding site (tgctg CCACA CACAC ACACA CACAC ACACA CACAC ACACA CACACA CACACA Ccctga) (34). Five additional nucleotides beyond the WT1 recognition site were included in the 5' and 3' ends of the sequence, shown above in small letters. The oligos were 5' biotinylated to ensure strong binding to PMP (Invitrogen Life Technologies, Burlington, ON, Canada). PMP-DNA complex was then collected and washed 3 times in 4°C PBS using a magnet to precipitate the beads to wash unbound DNA. Total cell lysate was then added to the clean PMP-DNA pellet and incubated for 1 hr at 37°C. PMP-DNA-protein complex was then collected using a magnet and consequently washed 3 times in 4°C PBS. The supernatant was discarded and the pellet was desalted using the ReadyPrep 2-D cleanup kit (Bio-Rad Laboratories, Hercules, CA). The protein pellet was then resuspended in rehydration sample buffer.

Western blot analysis and 2-D electrophoresis: Cells were harvested and homogenized with lysis buffer (10 mM Hepes, 2 mM $MgCl_2$, 15 mM KCl, 0.1 mM EDTA, and 0.15% Nonidet P-40, containing a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Samples were subjected to 7% SDS-polyacrylamide gel electrophoresis followed by blotting and immunodetection with antibodies. In some experiments 2-D electrophoresis was conducted by loading 200 µl of sample onto 7 cm IPG Strips pH 3–10 and electrofocused using the Protean IEF Cell (Bio-Rad Laboratories, Hercules, CA). Strips were then loaded onto Tris-HCl 4–15% gels and separated by electrophoresis. Gels were then analyzed by western blot

(18) or silver staining using the Amersham silver staining kit (Amersham Biosciences, Piscataway, NJ).

Molecular weight and isoelectric point estimation: To estimate molecular weights of spots detected by anti-WT1 antibodies from Western blot analysis of 2-D gels, we used the Odyssey Imaging System (Li-cor Biosciences, Nebraska, USA). 2-D electrophoresis was run with samples generated from either immunoprecipitation or DNA precipitation. Using molecular weight standards, the software estimates the molecular weight of a given spot. Using isoelectric point (pI) standards from Bio-Rad specific to our IPG strips an isoelectric point scale was designed as shown in Fig. 3.1C. The pI values for WT1 were estimated using this scale and also using the IgG heavy chain pI as a reference. Using molecular weight and pI data, mean ± standard error values were estimated (n = 3-4).

Subcellular fractionation: Subcellular fractionation was performed according to (35). Briefly, following stimulation A549 cells were harvested ($15x10^6$ cells), washed with cold PBS and resuspended in lysis buffer, subjected once to a freeze-thaw procedure, and kept on ice for 15 min. The supernatant was centrifuged at 14,000 g for 20 min and stored as cytoplasmic extract. The pellet was washed with lysis buffer, and nuclei were resuspended in 100 µl of extraction buffer (20 mM Hepes, 1.5 mM $MgCl_2$, 0.4 M NaCl, 20% glycerol, 0.2 mM EDTA, and 0.15% Nonidet P-40, with protease and phosphatase inhibitors) and left on ice for 30 min. The nuclear extract was recovered by centrifugation at 14,000 g for 20 min and stored in aliquots at –80°C for use in Western blot analyses. The nuclear extracts were tested for purity by

Western blot using antibody to the cytoskeleton protein α-tubulin and nuclear protein Oct-1.

Immunohistochemistry analysis: Histopathological material including sections of healthy adult lungs (n = 3) were obtained from the University of Alberta Hospital, Edmonton, Alberta, Canada. The study was approved by the Health Research Ethics Board, University of Alberta. Sample block preparation was performed by the University of Alberta Hospital, Department of Laboratory Medicine and Pathology. After fixation in 10% buffered formalin, lung tissue samples were embedded in paraffin and sections cut at 4 μm.

All steps were performed at room temperature (~20° C) in a humidified container to prevent dehydration of tissues. Sections were deparaffinized and rehydrated to water. Endogenous peroxidase was blocked with 30% hydrogen peroxide-methanol (1:4) for 10 min. Sections were covered with a 1/5 dilution of normal goat serum or normal horse serum (Vector Lab, Burlingame, CA) for 15 min to block non-specific binding sites. Serum solution was removed by gentle tapping of the slides and a previously optimized concentration (13 μg/ml for polyclonal and 26 μg/ml for monoclonal antibody) of primary antibody in antibody diluent (Dako, Via Real Carpinteria, CA) was immediately applied to the sections and incubated for 45 min. Unbound antibody was removed by three washes with PBS. Biotinylated anti-goat IgG (H+L) or biotinylated anti-mouse IgG (H+L) (Vector) secondary antibody was applied to each section for 30 min, followed by 20 min incubation with R.T.U. Horseradish Peroxidase Streptavidin (Vector). Slides were washed three times for 2 min with PBS and stained with 3,3'-diaminobenzidine chromogene (DAB) (BioGenex, San Ramon,

CA) for 5 min. After washing in water, samples were counterstained in Harris Hematoxylin (Sigma) for 2 min, dehydrated through an alcohol series and xylene and mounted in Cytoseal XYL medium (Richard-Allan Scientific, Kalamazoo, MI). Antibody localization was detected microscopically by brown staining. As negative controls we used two methods. For isotype controls we incubated sections with the same dilution of purified mouse IgG (Cedarlane, Hornby, ON, Canada) as primary antibody followed by incubation with corresponding secondary antibody for 30 min. For a second negative control we pre-incubated anti-WT1 goat antibody with an excess of specific blocking peptide (Santa Cruz), (1: 5) for 24 hr. The antibody-peptide mixture was applied to a set of sections instead of primary antibody and incubated for 45 min, followed by 30 min incubation with secondary antibody. To evaluate distribution of WT1, examination was performed by three independent observers using different magnifications: X100, X200, X400, X1000 and finally by a pathologist who was blinded to source of tissue and also to previous examinations.

Nitric oxide detection: The NO dye DAF-FM was used to assess NO formation. A549 cells 10^5 were cultured overnight in 96 well black plates (Corning Incorporated, Corning, NY). After stimulation with 10 ng/ml TNF and the NOS inhibitors L-NAME, L-NMMA and 1400W (see Fig. 3.1 for concentrations) (Cayman Chemical, Ontario, Canada), cells were incubated with 10 μM DAF-FM for 30 min and washed twice with fresh media. Cells were then allowed to rest for 30 min and fluorescence was measured using fluorescence plate-reader FLx800i (Biotech Instruments, Inc, Winooski, Vt) with excitation wavelength at 485 nm and detection at 516 nm.

WT1-small interfering RNA transfection: Small interfering RNA was used as previously described (36) with some modifications. WT1 mRNA was targeted using the sequence 5' AAGGACUGUGAACGAAGGUUU 3' in exon 8. The control sequence was generated by inverting two 4 nucleotide-long regions, shown in small letters 5' AAGG gtca TGAA aagc GGTTT 3'. A549 cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. Briefly, 10^6 cells/flask in a 75 cm^2 flask were transfected with 3 μg of WT1-siRNA or control siRNA diluted in 10 ml of OPTI-MEM (GIBCO, Invitrogen) and 25 μg/ml of Lipofectamine 2000. After a 4 hr incubation of cells with siRNA-lipofectamine 2000 complexes, medium was replaced with Dulbecco's Modified Eagle Medium (Invitrogen Life Technologies, Burlington, ON, Canada), and cells were maintained for a total of 24 hr. Harvested cells were used for RNA or protein extraction. In some experiments, cells were treated with 10 ng/ml of TNF during the last 12 hr of culture, and supernatants and media were frozen at –80°C for WT1 and MMP-9 detection respectively. Sham treatments contained 25 μg/ml lipofectamine.

Chromatin immunoprecipitation (ChiP): The technique was modified from Lueche and Yamamoto (37). The human LEC line A549 was grown to 90% confluence and treated for 10 min with 10 ng/ml TNF. Nine x 10^6 cells were used in 75 cm^2 flasks. Cross-linking was performed by adding freshly prepared buffer A (50 mM HEPES-KOH pH 8, 1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl, 11% formaldehyde). Formaldehyde was then quenched with 125 mM glycine for 5 min at 4°C. Cells were gently washed in PBS once, to remove glycine. Cells were scraped and collected with

10 ml PBS and harvested by centrifugation at 600g, 4°C for 10 min. Lysis was performed by incubating at 4°C for 10 min in 10 ml Lysis Buffer (50 mM HEPES-KOH at pH 7.4, 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10% Glycerol, 0.5% NP-40 and 0.25% Triton X-100). The buffer was supplemented with 1 mM PMSF and 1 μg/ml protease inhibitor cocktail (Upstate Cell Signaling Solutions, VA, USA). Nuclei are collected by centrifugation at 600g for 5 min and washed in 8 ml of Washing Buffer (10 M Tris-HCl pH 8, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl), supplemented with 1 mg/ml of protease inhibitor cocktail, for 10 min at room temperature. Samples were collected at 600g for 10 min and resuspended in 1.5 ml RIPA Buffer (10 mM Tris-HCl pH 8, 1 mM EDTA, 0.5 mM EGTA, 140mM NaCl, 5% Glycerol, 0.1% Sodium Deoxycolate, 0.1% Sodium dodecyl sulfate (SDS), 1% Triton X-100) supplemented with protease inhibitors. Samples were sonicated (F60, Fisher Scientific, Ottawa, ON, Canada) in nine 10 sec intervals, at 20% maximum output, keeping the tube on ice at all times with 1 min intervals between each sonication. This sonication procedure yielded DNA average fragment sizes of 500pb. Lysates were then cleared by centrifugation at 16 000g for 15 min at 4°C. Twenty μl of samples were saved as input. Thus input samples were used as a positive control in these experiments. Immunoprecipitation was performed using the ChiP assay kit (Upstate Cell Signaling Solutions, VA, USA). MMP-9 PCR primers were 5' CTGCGGGTCTGGGGTCTTGC 3' and 5' CGCTCCTGTGACCCCACCCC 3' with expected amplicon size of 197 bp. IL-13 was used as a control for WT1 binding. IL-13 primers were 5' CTC TTT CCT TTA TGC GAC ACT GG 3' and 5' CAT AGG

CTG GGT GGC TTG TGG 3' with expected amplicon size of 151 bp. PCR conditions were 63°C for annealing temperatures and 30 cycles.

Confocal microscopy: Cells were grown on glass coverslips overnight at 37°C. Cells were stimulated with TNF (10 ng/ml), washed with PBS and fixed with 4% paraformaldehyde at room temperature. After permeabilization with 0.2% Triton X100, nonspecific binding was blocked with 10% FBS plus 3% BSA. Cells were incubated overnight at 4°C with monoclonal antibodies diluted 1:50 in blocking buffer, washed three times with PBS and incubated with Rhodamine Red-X - conjugated secondary antibody (1:50) for 2 hr at room temperature. DAPI was used as a nuclear marker. Coverslips were mounted on glass slides and cells were examined with a confocal microscope (Olympus Fluoview, FV 1000).

Transcription factor-DNA interaction *in silico* analysis: The MMP-9 promoter was studied using *in silico* tools: Transcription Element Search System (TESS; http://www.cbil.upenn.edu/tess/) and MatInspector (http://www.genomatix.de/). These softwares were also used to study the mucin genes MUC2, MUC5AC and MUC5B.

Statistics: Results are means ± S.E. of at least three independent experiments. They were analyzed using one-way analysis of variance, and when significant differences were found, the multiple comparison Tukey-Kramer test was used (GraphPad InStat). Values where $P \leq 0.05$ were considered statistically significant. The *n* values in figure legends represent independent experiments.

4. Results

TNF induces iNOS-mediated MMP-9 expression and enzyme activity.

Since we previously observed that NO mediates MMP-9 gene expression in rat vascular smooth muscle cells (18), we tested whether NO-mediated regulation of MMP-9 also occurs in human LEC. A549 cells were stimulated with TNF (10 ng/ml) and NO production, iNOS and MMP-9 expression were investigated. TNF induced significant production of NO after 12 ($p < 0.05$) and 24 hr ($p < 0.01$) as detected by 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) (Fig. 3.1A). This effect was blocked by the NOS inhibitors 1400W (100 μM), L-NAME (300 μM) and L-NMMA (300 μM) (Fig. 3.1A). Time course for MMP-9 expression indicated a significant upregulation after 12 hr TNF treatment (Fig. 3.1B). Protein and mRNA iNOS expression was studied at 10 min, 6, 12, 24 and 48 hr after TNF treatment (Fig. 3.1B). From these data 12 hr was selected as the optimum point for MMP-9 activity using gelatin zymography. Following optimization of PCR reactions for the log phase of amplification (see methods) we found that MMP-9 and iNOS mRNA levels were undetectable (30 cycles) in resting (sham) A549 cells (Fig. 3.1C). After 10 min of TNF stimulation, iNOS and MMP-9 mRNA signals were significantly upregulated (Fig. 3.1C, 0 μM 1400W). Furthermore, 1400W blocked TNF-induced MMP-9 gene expression in a concentration dependent manner (0, 2 and 20 μM), but did not block iNOS mRNA expression. These data show that 1400W blocks iNOS activity, but not iNOS expression itself. Thus iNOS activity is required for MMP-9 gene expression.

To investigate the role of iNOS activity in the induction of MMP-9 enzyme activity, gelatin zymography analysis of the media (without FBS) used to grow A549, was performed. We detected low levels of MMP-9 activity in resting (sham) A549 media. These levels were significantly upregulated (4.5 fold) after 12 hr of TNF treatment

(Fig. 3.1F, H). The NOS inhibitors L-NAME and 1400W significantly reduced MMP-9 activity in a concentration dependent manner (Fig. 3.1E, G). These data suggest that NO is an important upregulator of MMP-9 gene expression and activity in human LEC.

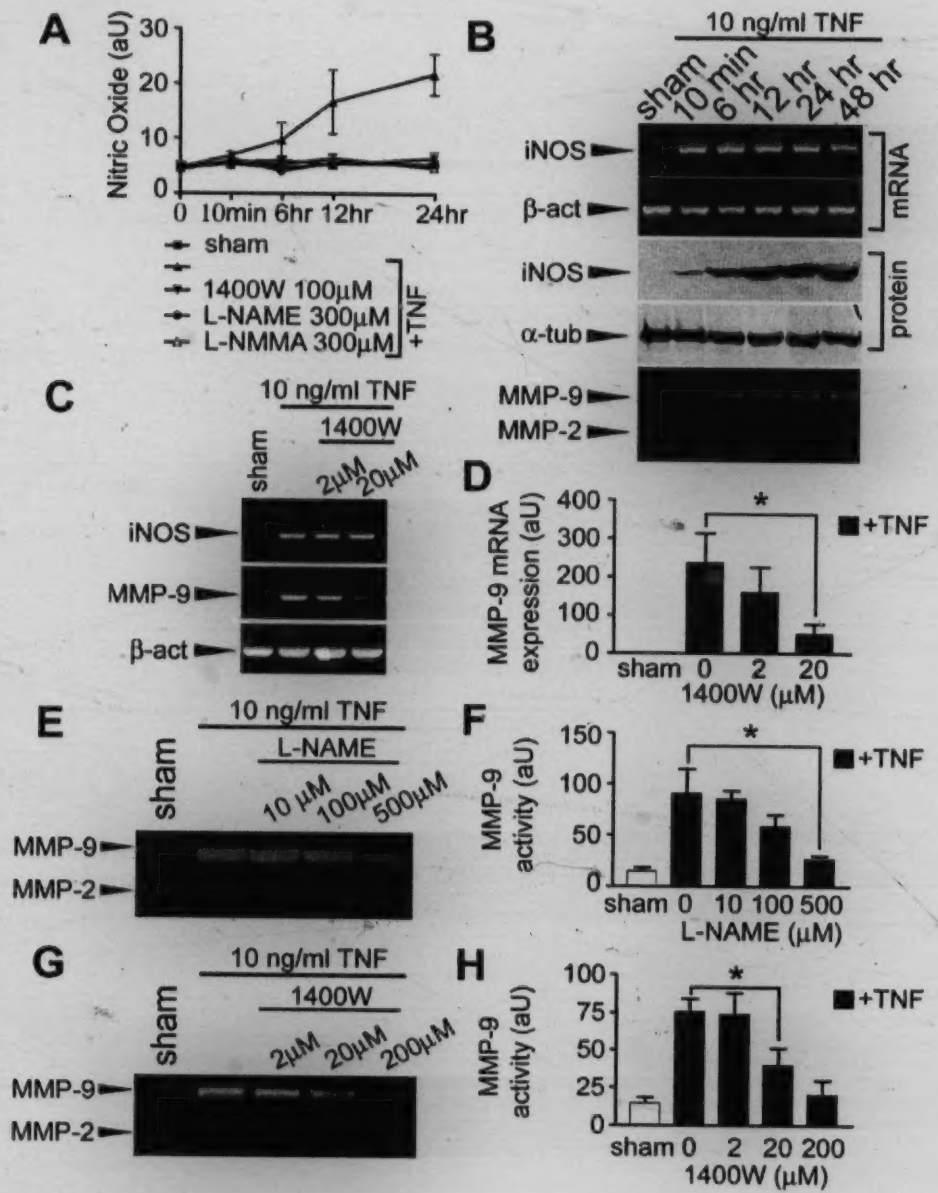


Figure 3.1: NO regulates MMP-9 gene expression in human LEC A549. (A) A549 cells were stimulated with TNF in the presence of the NOS inhibitors 1400W, L-NAME and L-NMMA. NO release was measured using the NO specific dye DAF-FM ($n = 4$). (B) TNF Time-course shows iNOS expression from 10 min to 48 hr after treatment and MMP-9 activity. (C) RT-PCR was used to study MMP-9, iNOS and β-actin gene expression ($n = 3$). (D) Densitometric analysis of (C) plotted as an MMP-9/β-actin ratio representing MMP-9 expression in arbitrary units (aU). (E, G) MMP-9 protein expression and activity was studied using gelatin zymography ($n = 4$). (F, H) Densitometric analysis was performed and the ratio MMP-9/MMP-2 plotted as % of control of MMP-9 activity. Statistical significance: $p < 0.05$ (*); $p < 0.01$ (**).

PKA and sGC are involved in MMP-9 gene regulation

Using rat vascular smooth muscle cells we previously showed that NO regulates MMP-9 through a sGC and cGMP-dependent pathway (18). To assess this pathway in human LEC, we used N^G-monomethyl-L-arginine (ODQ) a sGC inhibitor. ODQ significantly downregulated MMP-9 activity at 0.5 and 5 μM (Fig. 3.2 A and B), consistent with our findings in rat smooth muscle (18). Thus we studied downstream targets of sGC that could be involved in this signaling pathway.

Active sGC produces cGMP and the latter can mediate gene regulation through the activation of cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinase (PKA) (27-29). To test the role of PKG and PKA in the signal transduction of this pathway we used the membrane soluble PKG and PKA specific inhibitors AD-3 and myrPKA respectively. The PKG inhibitor AD-3 did not affect TNF-induced

MMP-9 upregulation (Fig. 3.2C). The PKA inhibitor myrPKA however significantly reduced $p<0.01$) the MMP-9 activity in a concentration dependent way (Fig. 3.2D).

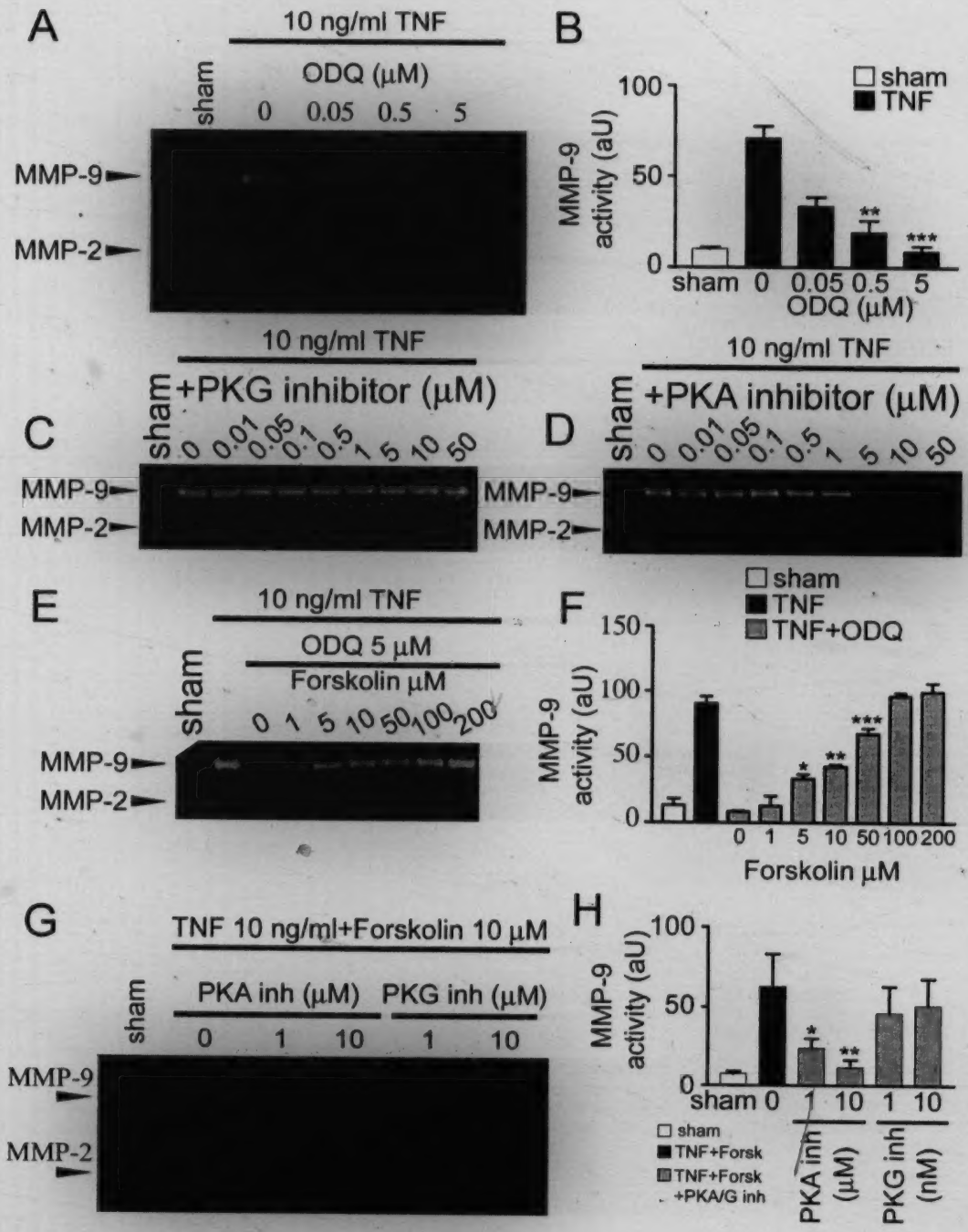


Figure 3.2: MMP-9 gene expression is regulated by sGC and PKA. (A) A549 were stimulated with TNF in the presence of the sGC inhibitor ODQ (n = 3). Densitometric analysis of the zymography gels was performed (B). The effect of PKG (AD-3) and PKA (myrPKA) inhibitors on the TNF-dependent upregulation of MMP-9 was studied through zymography. (E) A549 cells were stimulated with TNF in the presence of ODQ. The effect of increasing concentrations of Forskolin was studied under these conditions. (F) Densitometric analysis of (E). (G) A PKA inhibitor (myrPKA) and a PKG inhibitor (AD-3) were used to study the role of PKA and PKG downstream of sGC. (H) Densitometric analysis of (G). Statistical significance: $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$.

To investigate further the role of PKA in the regulation of MMP-9 production we used the adenylate cyclase agonist forskolin to activate PKA. In this case we would expect forskolin to reverse the ODQ-induced inhibition of MMP-9 activity. We found that the ODQ-dependent reduction of MMP-9 activity was prevented in a concentration dependent manner by forskolin (Fig. 3.2E). Moreover, recovery of MMP-9 activity was blocked by a PKA inhibitor (myrPKA) but not a PKG inhibitor (Fig. 3.2G). These data supported the hypothesis that PKA mediates the NO-dependent regulation of MMP-9, perhaps through PKA translocation to the nucleus and phosphorylation of transcription factors that regulate gene expression.

Comparative genomic analysis of MMP-9

Comparative genomic analysis is a powerful method for identifying sequence conservation over evolution and as such putative regulatory elements (38). Figure

3.3A represents a comparative analysis of the MMP-9 gene across several mammalian species and demonstrates significant conservation of the 5' region. Indeed, -321/-21 (relative to the ATG) was highly identical from human to cow (75%, upper), rat (72%, middle) and mouse (67%, lower). Figure 3.3B demonstrates sequence alignment of these species and reveals the presence of a CA repetitive element. While *in silico* analysis using Transcription Element Search System (TESS; http://www.cbil.upenn.edu/tess/) and MatInspector (http://www.genomatix.de/) revealed the presence of numerous putative transcription factor binding sites, the most highly conserved sequence was the [CA]21 repeat, which was predicted to contain multiple binding sites for a transcriptional repressor called WT1. These results indicated WT1 may be an important transcriptional regulator of MMP-9.

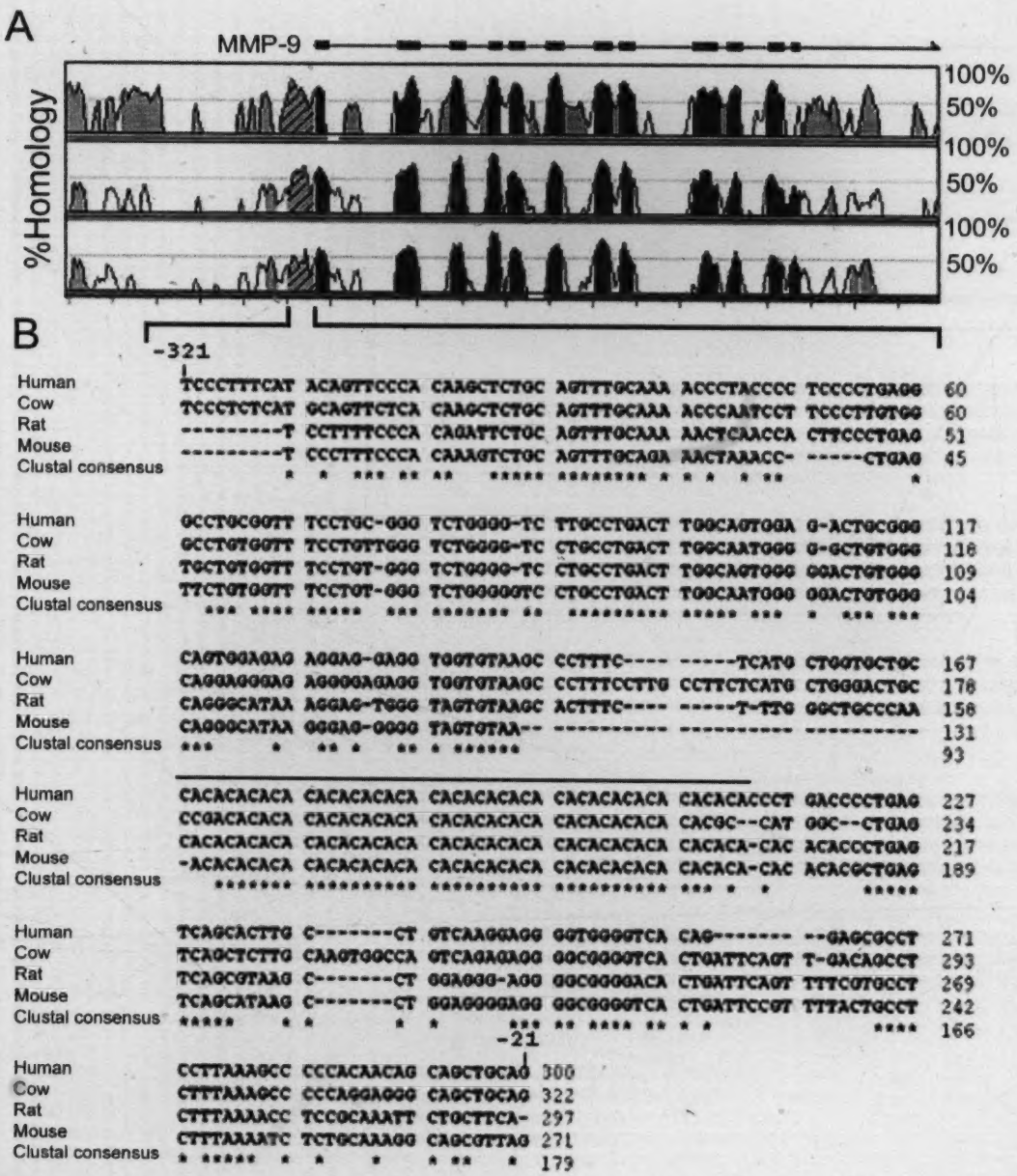


Figure 3.3: Conservation of MMP-9 across multiple species. (A) Comparative analysis of MMP-9 using VISTA software (http://pipeline.lbl.gov/cgi-bin/gateway2) demonstrates the profile of conservation across the MMP-9 gene from human to cow (top plot), rat (middle plot) and mouse (lower plot). Peaks correspond to highly

conserved sequences. Black shading corresponds to peaks in coding sequences and light shading corresponds to peaks in non-coding sequences. (B) Multiple sequence alignment demonstrates conservation of a $CA_{(21)}$ repetitive element (over-lined)

WT1 is expressed in human lung epithelial cells.

Given that WT1 may participate in transcriptional regulation of MMP-9 and the fact that LEC are a major source of MMP-9, we investigated whether these cells express WT1. Using RT-PCR analysis, we found that human LEC lines A549, HS-24, BEAS-2b, as well as cultures of primary normal human bronchial epithelial cells (PBEC) expressed WT1 mRNA (Fig. 3.4A). Because cell type-specific expression of exon 5 of WT1 has been reported (39), we studied positive and negative isoforms of WT1 in LEC. Primers binding to exon 4 and 6 were designed to detect differential expression of exon 5. The isoform lacking exon 5 is 51 bp shorter (see materials and methods). All LEC described above co-expressed WT1 isoforms with and without exon 5. The two RT-PCR bands of 412 and 361 bp (Fig. 3.4A) were cloned and the sequence identity was confirmed to be 100% homologous to WT1 splice variants with and without exon 5.

To study WT1 protein expression, A549, HS-24 and BEAS-2b cell lysates were analyzed by Western blot using the monoclonal antibody Ab-1 (Fig. 3.4B). A single band at 54 kDa was detected in all three cell lines. WT1 was immunoprecipitated from A549 cell lysate with the monoclonal antibody F-6, separated by 2-D electrophoresis and analyzed by Western blot using Ab-1 (Fig. 3.4C). A single spot was detected at the expected molecular weight of 54 kDa (Fig. 3.4C *i*). Three

additional spots were also detected and confirmed to be IgG heavy chain by mass spectrometry sequence analysis (Fig. 3.4C *ii*).

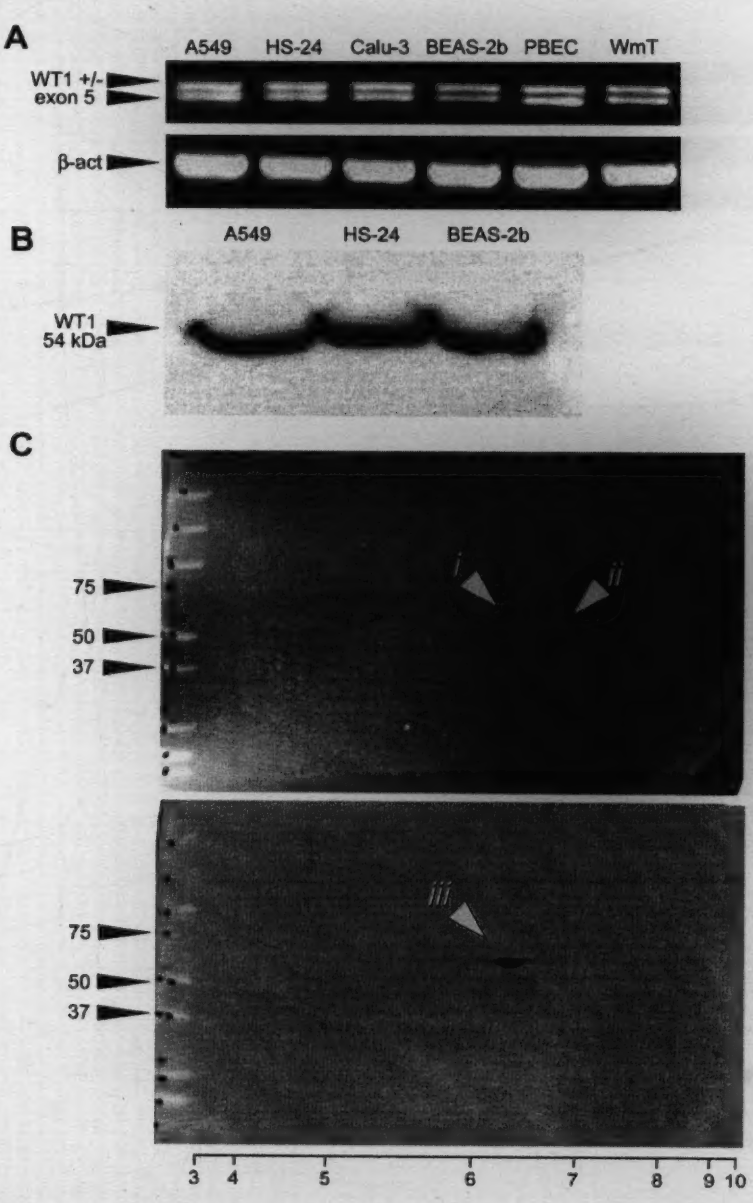


Figure 3.4: Human lung epithelial cells express WT1. (A) RT-PCR analysis of three lung epithelium derived carcinoma cell lines (A549, HS-24 and Calu-3), a human bronchial epithelial cell transformed by adenovirus 12-SV40 (BEAS-2b) and

primary bronchial epithelial cells (PBEC) express two WT1 mRNA variants with and without exon 5. A Wilms' tumor positive control (WmT) was also examined (n = 4). (B) Cell lysates from A549, HS-24 and BEAS-2b were analyzed by Western blot (n = 3-6). (C) Left blot corresponds to A549 cell lysate immunoprecipitated with the monoclonal antibody F-6, separated by 2-D electrophoresis and analyzed by Western blot with the monoclonal antibody Ab-1 (n = 5). Molecular weight markers at 75, 50 and 37 kDa are shown. A pI bar (pH 3 to 10) was added for reference purposes. (C *i*) WT1 was detected at 54 kDa and pI 6.5. (C *ii*) IgG heavy chain originated from F-6 was detected at pI 7 and 52 kDa. (C *iii*) Right blot corresponds to precipitation of WT1 using its DNA binding site (see methods), separation by 2-D electrophoresis and later detected by Western blot using Ab-1 (n = 4).

To determine if WT1 is also expressed in normal LEC, immunohistochemical analysis of sections of human lung biopsies was examined. Immuno-reactivity for WT1 was associated with the epithelial cells using two different antibodies (Fig. 3.5). WT1 displayed both, nuclear and perinuclear subcellular localization (Fig. 3.5C).

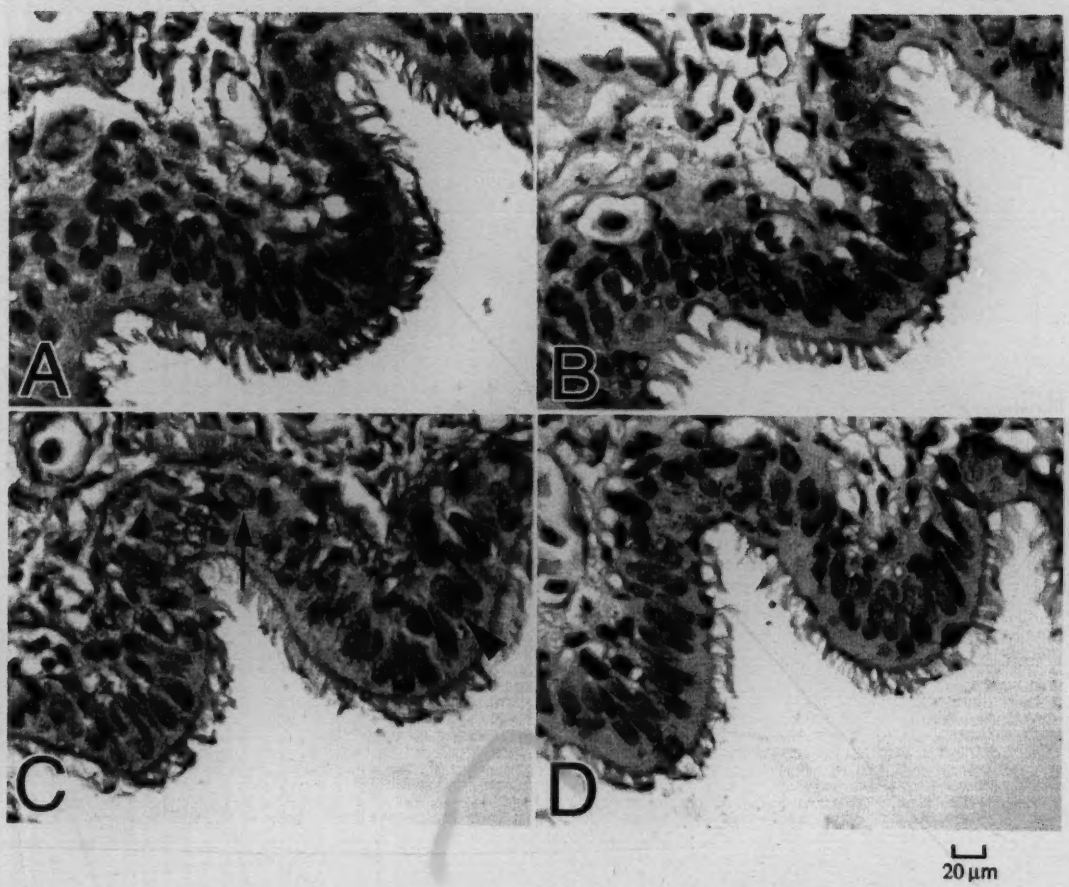


Figure 3.5: WT1 is expressed in the nuclei of human lung epithelial cells. (A) Immunohistochemistry analysis was performed in normal tissue from adult human lung biopsies. The tissue was stained with the anti-WT1 polyclonal antibody N20. (B) N20 was pre-incubated with its peptide blocker N20-P prior to staining tissue samples. (C) The anti-WT1 monoclonal antibody F-6 and its IgG_1 isotype control (D) were also used to study WT1 expression and its distribution. Arrow points to a nucleus displaying perinuclear WT1 localization. The majority of nuclei showed positive granular staining to WT1 (arrowhead).

WT1 binds to the MMP-9 promoter in vitro

Given our observation that there is a WT1 binding site on the MMP-9 promoter, we designed a DNA-paramagnetic particle (PMP) construct with the MMP-9 promoter sequence containing the putative WT1 site (-142 to –84) to isolate WT1 (see methods). Following incubation of the DNA-PMP with A549 cell lysate, WT1-DNA-PMP complex was precipitated using a magnet and resolved by 2-D electrophoresis. Western blot analysis using the monoclonal antibody Ab-1 detected a spot of 54 kDa (Fig. 3.4C *iii*). A total of four gels using immunoprecipitation to isolate WT1 and three gels using the DNA-PMP approach were used as independent experiments to estimate the Mr of WT1 from LEC (see methods). These two groups gave similar Mr for WT1 (53.2±4.0 kDa and 56±0.1 kDa respectively, $p>0.05$). The molecular weight mean for both groups was 54.7±3.4 kDa, consistent with the WT1 reported molecular weight of 54 kDa. The pI values for these groups were estimated to be 6.4±0.1 for samples obtained through immunoprecipitation and 6.5±0.2 through DNA isolation. The combined pI of both groups was 6.5±0.1. These data demonstrate the presence of a WT1 isoform of 54 kDa and pI 6.5 in human LEC and that this protein can be precipitated with the sequence of –142 to –84 of the MMP-9 promoter. Collectively, these results indicate that WT1 is expressed in LEC and at least in vitro, WT1 binds to the MMP-9 promoter.

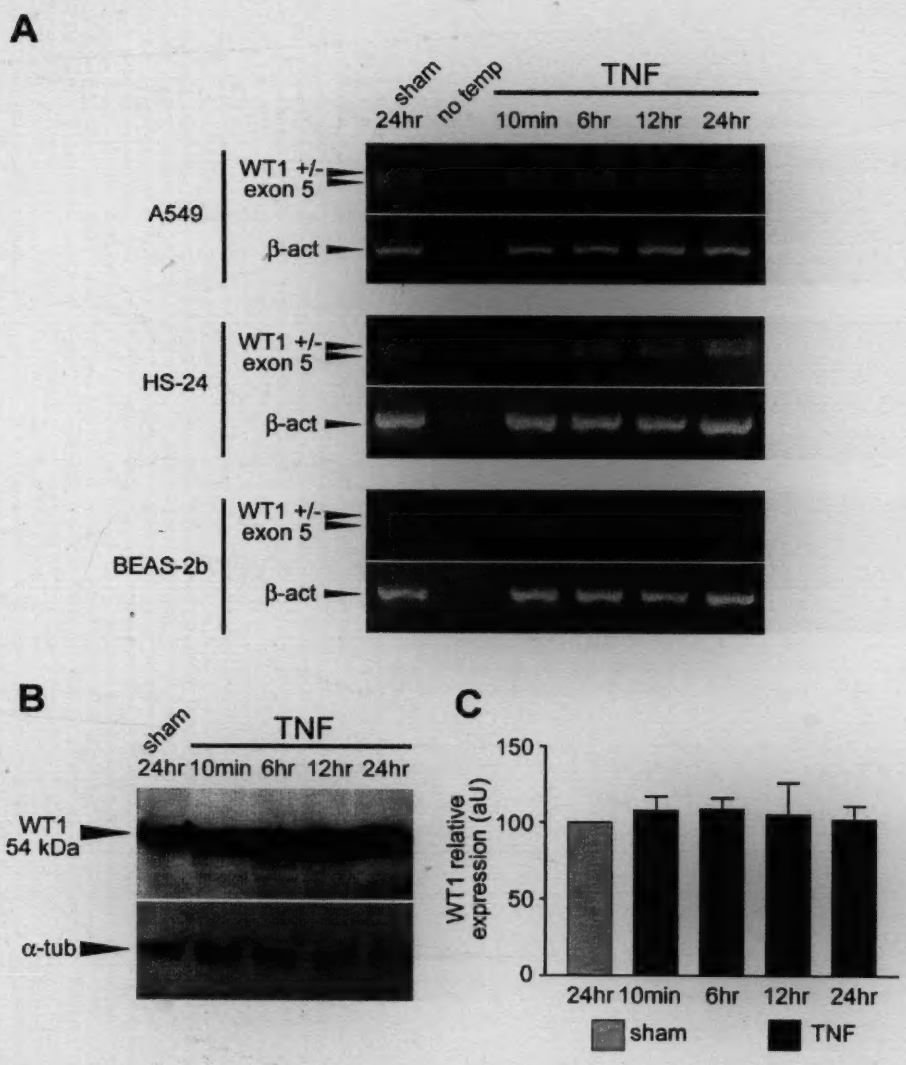


Figure 3.6: TNF does not affect WT1 mRNA and protein expression. (A) RT-PCR analysis shows WT1 mRNA expression in human LEC A549, HS-24 and BEAS-2b following 10 ng/ml TNF treatment from 10 min to 24 hr. β-actin was used as the internal positive control. A no template control (no temp) was also loaded. (B) Cell lysate from A549 was extracted after treating cells with TNF for 10 min to 24 hr (n = 3). The loading control α-tubulin (α-tub) was used. (C) Densitometric analysis

of (B) was performed and a WT1/ α-tubulin ratio was used to calculate % of control and plotted as WT1 expression.

WT1 subcellular localization is regulated by NO

We next examined whether upregulation of MMP-9 expression in response to proinflammatory stimuli such as TNF could be associated with altered WT1 expression. RT-PCR using cDNA extracted from A549, HS-24 and BEAS-2b stimulated with TNF (10 ng/ml) revealed that WT1 mRNA levels did not change at time points from 10 min to 24 hr (3. 6A). Similar results were obtained using a more potent proinflammatory stimulus consisting of PMA, IFNγ and LPS (see methods) that we previously showed induces expression of iNOS, cyclo-oxygenase-2 (COX-2) and MMP-9 (18) (data not shown). Furthermore, WT1 protein levels did not change under similar conditions (Fig 3.6C).

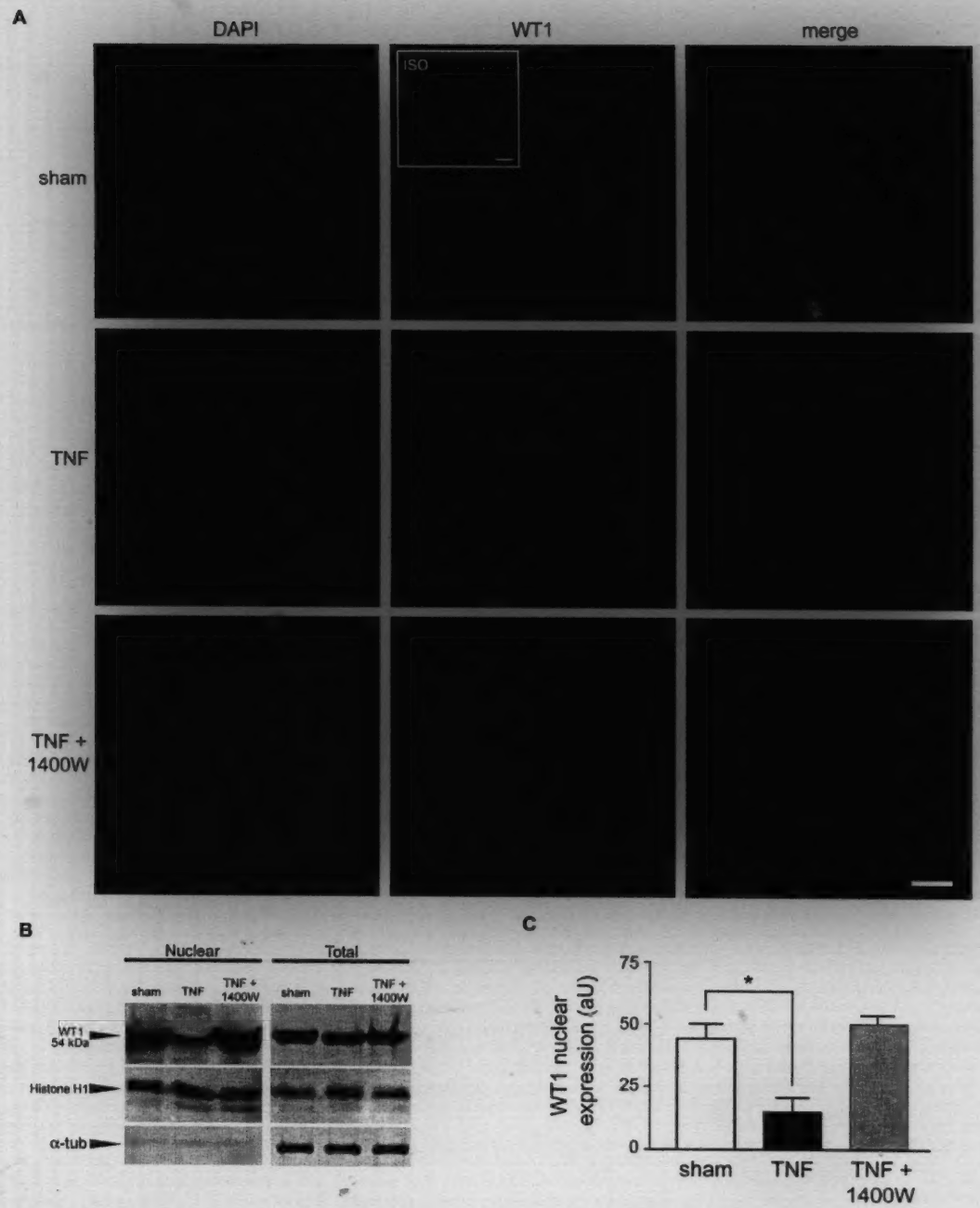


Figure 3.7: TNF induces WT1 translocation from the nucleus to the cytosol. (A) Confocal microscopy of A549 cells was performed using the anti-WT1 antibody Ab-1 (red). Nuclei were stained with DAPI (green). Co-localization was observed by

merging the green and the red channels (yellow). The isotype control (iso) is shown for the sham treatment. Bars indicate 10 μm (n = 5). (B) A549 cells were treated as described in the text followed by purification of the nuclear fraction and Western blot analysis (n = 3). The nuclear specific loading control histone H1 was used to perform the densitometric analysis. The nuclear localization control α-tubulin (α-tub) was measured. (C) A ratio WT1/H1 was used to calculate WT1 nuclear expression in arbitrary units (aU). Statistical significance: $p < 0.05$ (*).

Since there was no apparent change in relative abundance of WT1, we assessed the possibility that intracellular localization of WT1 may change upon stimulation. A549 cells were treated with TNF and analyzed using confocal microscopy using a monoclonal antibody (Fig. 3.7). In sham treated cells (sham) the nuclear stain DAPI co-localized with WT1 signal (merge). After 10 min of TNF (10 ng/ml) treatment, the WT1 signal (red) was detected only in the perinuclear region (Fig 3.7A). Since NO and PKA can regulate MMP-9 expression and WT1 has been shown to be regulated by targets downstream of NO, such as PKA, we examined whether the apparent translocation of WT1 following TNF treatment was NO-dependent. Indeed, pre-incubating the cells with the iNOS inhibitor 1400W (100 μM) prior to TNF stimulation blocked the alteration in the apparent location of WT1 at 10 min (Fig 3.7A), as well as at 1, 3 and 6 hr (data not shown).

Additionally, evidence confirming loss of WT1 from the nuclear milieu is provided by Western blot (Fig. 3.7B). As indicated by the confocal data (Fig 3.7A), nuclear WT1 signal was significantly lower in TNF-treated cells and when cells were pre-

treated with the NO synthase inhibitor 1400W, WT1 remained in the nucleus (Fig. 3.7A). Together these results provide strong evidence that the levels of nuclear detectable WT1 drop in response to TNF in a NO-dependent mechanism.

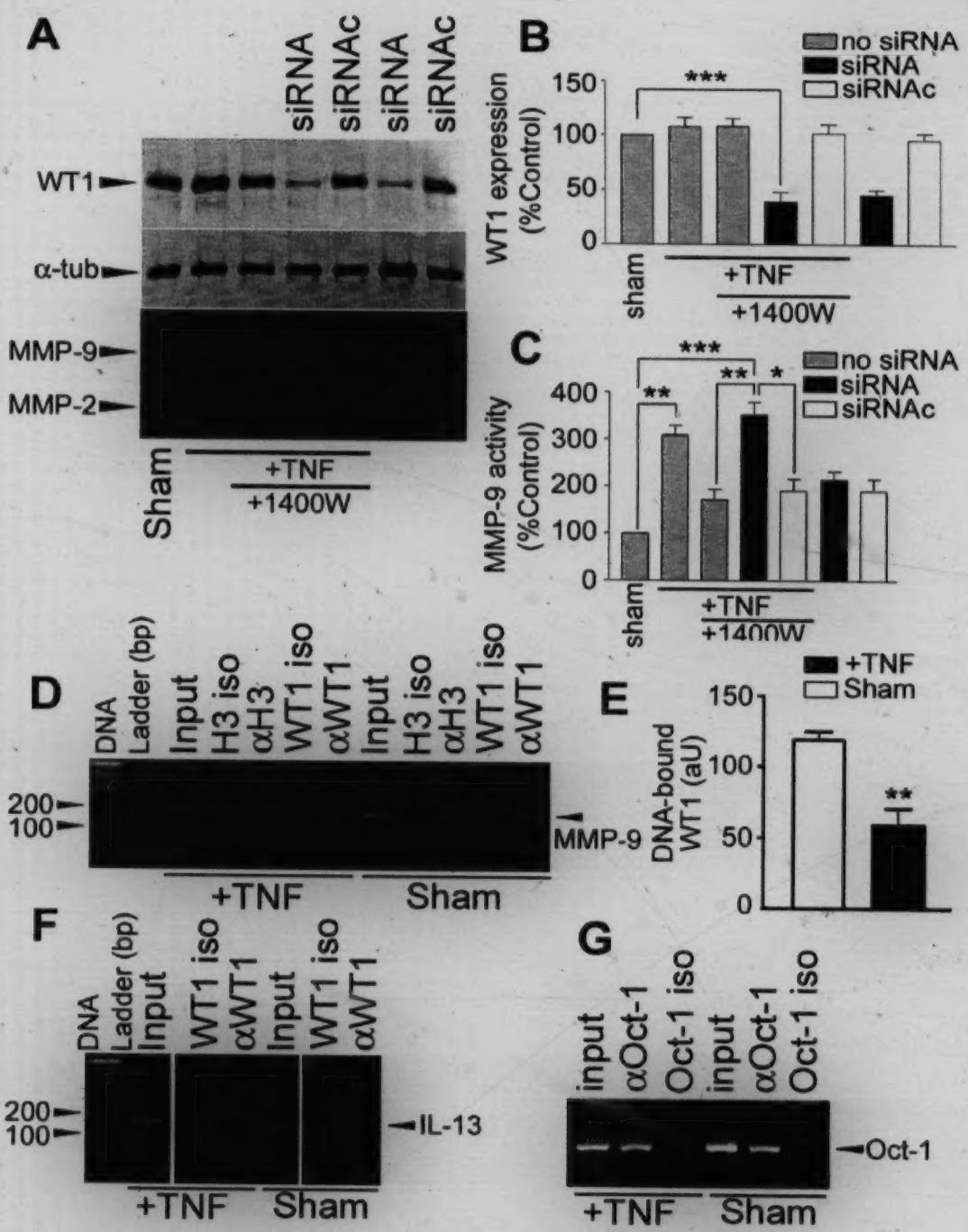


Figure 3.8: WT1 functions as a MMP-9 repressor. (A) A549 cells were treated with siRNA targeted to WT1 and WT1 protein expression was detected by Western

blot. Sham treated cells were incubated with lipofectamine (sham). Controls treated with TNF or TNF plus 1400W but no siRNA were included (lane 3 and 4). The siRNA control sequence was also included (siRNAc). The loading control α-tubulin (α-tub) was measured (n = 5). MMP-9 protein expression and activity was studied using gelatin zymography. (B) Densitometric analysis of (A) was carried out and used to estimate the WT1/α-tubulin ratio and plotted as % of control of WT1 expression (n = 4). (C) Densitometric analysis for zymograms was performed and the ratio MMP-9/MMP-2 plotted as % of control of MMP-9 activity (n = 5). (D) Chromatin immunoprecipitation analysis was performed (n = 3). Input DNA was used as the positive control. Immunoprecipitation was performed using an anti-histone-3 (αH3) or anti-WT1 (αWT1) and their respective isotype controls (H3 iso) and (WT1 iso). PCR primers were used to detect MMP-9 (upper gel) and IL-13 (lower gel). (E) Densitometric analysis of (D) was carried out and the αH3/αWT1 ratio was plotted as *in vivo* DNA-bound WT1. (G) Oct-1 binding was measured as a constitutive promoter control. Statistical significance: $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

WT1 depletion increases MMP-9 expression.

WT1 is a transcription factor that can act as a gene repressor (30-32). As such, we reasoned that under conditions where WT1 is shuttled to the cytosol, it cannot fulfill its repressor functions resulting in upregulation of MMP-9. To test this postulate, A549 cells were depleted of WT1 using siRNA and cells were treated with TNF in the presence or absence of 1400W. Figures 3.8A and B represent western blot analysis of A549 cell lysate and demonstrate the significant reduction of WT1 levels

in cells treated with siRNA for WT1 (39%) compared to sham treated cells or those treated with an oligonucleotide containing two inverted sequences of four nucleotides (siRNAc, lane 5). Furthermore, Figure 3.8C shows that the NO-mediated increase in MMP-9 activity observed following TNF treatment is WT1 dependent. Indeed in cells depleted of WT1, treatment with TNF and 1400W resulted in MMP-9 activity similar to that observed for WT1 expressing cells following TNF stimulation. In the absence of 1400W, siRNA treatment alone did not increase MMP-9 enzyme activity (data not shown).

WT1 binds to the MMP-9 promoter in vivo and binding is regulated by TNF

To confirm *in vivo* binding of WT1 to the MMP-9 promoter, we performed chromatin immunoprecipitation (ChiP) analysis of A549 cells. Immunoprecipitation was performed with antibodies against histone H3 (αH3), WT1 F-16 (αWT1) and the appropriate isotype controls. Primers designed to amplify the MMP-9 promoter generated a product from sonicated DNA prior to immunoprecipitation (input) as well as sham- or TNF-treated cells immunoprecipitated with αH3. However, the product generated with DNA immunoprecipitated with αWT-1 following TNF-treatment appears to be reduced compared to sham-treatment, when corrected against αH3 (n = 3, Fig. 3.8D and E). No bands were amplified with primers for the IL-13 promoter which lacks a WT1 binding site. The constitutive promoter of the gene thioredoxin reductase 1 (40) was used to detect binding of the constitutive factor Oct-1. Although the input control for Oct-1 was lower in TNF treated (Fig. 3.8G) the relative to input Oct-1 signal was not reduced. These findings support our hypothesis of WT1 as a transcriptional repressor of MMP-9.

5. Discussion

NO is an important second messenger that is produced at high concentrations in conditions such as asthma and COPD. We have identified WT1 as a novel target by which NO exerts regulation of MMP-9 gene expression.

Human LEC express MMP-9 in resting conditions and this expression was shown to be significantly upregulated with TNF treatment. We showed that TNF-induced upregulation of MMP-9 was NO-dependent in A549. Although detectable NO production after 10 min is not significantly different than at time 0 using the DAF assay (Fig. 3.1A), intracellular iNOS blockade using the specific iNOS inhibitor 1400W reduced MMP-9 mRNA expression but not iNOS (Fig. 3.1C). Further more 1400W also blocked the TNF-induced translocation event of WT1 (Fig. 3.7). Interestingly 1400W at 20 μM but not L-NAME at 100 μM significantly reduced MMP-9 activity (Fig. 3.1E, G) suggesting that the TNF-induced NO production is iNOS dependent and not from basal sources. Collectively, these data indicates that within 10 min of TNF activation iNOS activity is sufficient to induce WT1 translocation to the cytosol an event that promote the formation of MMP-9, which accumulates in the media to measurable levels after 12 hr (Fig. 3.1B).

Downstream of NO, sGC was shown to mediate this pathway through activation of PKA (Fig. 3.2). In this study we have extended our observations in rat vascular smooth muscle cells (18) into a new human cell compartment, the lung epithelium. In addition we have identified PKA as an important target of NO in the regulation of MMP-9 mRNA expression and enzymatic activity. Recently PKA has been shown to activate MMP-9 in human ovarian epithelium (41) and we have confirmed this

findings in human LEC. WT1 has been shown to be regulated by PKA through phosphorylation of its serine residues (26, 34, 42) in agreement with our findings.

WT1 expression was not restricted to carcinoma cell lines such as A549, HS-24 and Calu-3, but also present in primary cell cultures (PBEC) and in BEAS-2b, a virus immortalized human LEC line. Additional evidence supporting the expression of WT1 in human LEC was obtained from immunohistochemical analysis of normal adult lung (Fig. 3.5). In addition to demonstrating the expression of WT1 in human tissue these data indicate that WT1 may change its subcellular micro-localization *in vivo*.

A number of different WT1 splice variants have been described in the literature. Exon 1, exon 5 and exon 10 are either spliced or modified before a mature mRNA is generated (39). By designing our RT-PCR primers to recognize exon 4 and exon 6, we were able to amplify PCR products with and without exon 5. Different cell types may alternatively express WT1 isoforms with or without exon 5, whereas other cell types may co-express both isoforms (39). It has been reported that exon 5 might play a role in repressor functions of WT1 (43). Our results indicate that the isoform variant containing exon 5 is more abundant at the mRNA level (Fig. 3.4A), however both isoform mRNA types are co-expressed in all LEC tested. In our studies we were able to identify only one WT1 isoform at the protein level. The 54 kDa isoform is generated from the full size transcript encoding all 10 exons, which was the most abundant isoform detected by PCR. We were able to detect only one band at 54 kDa using western blot and only one spot using 2-D electrophoresis. Thus the 54 kDa

WT1 isoform seems to be the major WT1 variant in LEC and the isoform that undergoes changes in cellular micro-localization.

Stimulation of A549, HS-24 and BEAS-2b with 10 ng/ml TNF for 10 min to 24 hr did not change WT1 mRNA expression levels or protein levels (Fig. 3.6). However, this treatment induced a change in localization of WT1 indicating potential translocation of WT1 from the nucleus to the perinuclear area (Fig. 3.7). Interestingly, this response to TNF was blocked with the NOS inhibitor 1400W, suggesting that NO mediates TNF effects in this pathway.

To test whether NO regulates MMP-9 gene through WT1, siRNA was used to knockdown WT1 (Fig. 3.8). We found that WT1 levels were significantly reduced with the siRNA treatment and that WT1 knockdown resulted in MMP-9 upregulation (Fig. 3.8) even in the presence of 1400W, indicating that NO was controlling the MMP-9 gene activity through regulation of WT1 micro-localization. Thus we have shown with carefully optimized, semi-quantitative RT-PCR, in combination with western blot analysis and with MMP-9 enzyme activity, that MMP-9 is regulated by WT1. The evidence we provide clearly supports the model in which WT1 acts as a MMP-9 gene repressor, regulated by PKA.

A powerful technique that studies *in vivo* binding of transcription factors to their specific DNA binding sites, ChiP, was used. We found that TNF treatment significantly reduces the amount of WT1 that binds to the MMP-9 promoter (Fig. 3.8). To confirm the specificity of WT1 binding to the MMP-9 promoter, we used primers for the IL-13 promoter that is known to lack WT1 binding sites (Fig. 3.8D). Collectively these data indicate that WT1 is a repressor of the MMP-9 gene in human

LEC and in conditions of high NO, WT1 translocates to the perinuclear region thus de-repressing the MMP-9 gene.

Our findings add the MMP-9 gene to a growing number of growth related genes that have been reported to be repressed by WT1, including platelet-derived growth factor (PDGF A-chain), insulin-like growth factor II (IGF II) and early growth response 1 (EGR-1) (30-32).

In contrast to our results, using rat embryo fibroblasts and a transient transfection approach, Himelstein *et al* found that deletion of the WT1 binding site in the MMP-9 promoter inhibited MMP-9 promoter activity (44). In this study the authors created a promoter sequence lacking the WT1 binding site. The WT1 binding site is 42 nucleotides long and it is located near the TATA box at position -131 bp. It is possible that deletion of 42 bases in this region would result in a frame shift that would interfere with the functionality of the promoter, thus explaining contrasting results found by the authors. Additionally, the approach that they used with transient transfection has a number of limitations e.g., uncontrolled plasmid copy number and no genomic integration. By contrast, in human LEC BEAS-2B, Wu. T. *et al.* found that the same sequence had inhibitory properties in the cytosolic phospholipase A2 gene (45), raising the possibility that there is differential expression of WT1 in different cell types. Interestingly, we have found that human lymphocytes express WT1 and that NO reduces WT1 levels rather than changing WT1 subcellular localization. These findings support the view that NO can regulate WT1 at multiple levels (e.g. expression and localization) and that these mechanisms are cell specific (in progress).

Using a novel homologous recombination technique Yan C.H. *et al* reported that the MMP-9 gene was potentially controlled by a gene repressor and that this phenomenon was not apparent through transient transfection (46), explaining contradictory results found by Himelstein *et al*. Our findings confirm this proposal and provide new insight into the regulation of the MMP-9 gene.

The WT1 gene is located at 11p13. At position 11p15 the mucin family of genes MUC2, MUC5AC, MUC5B and MUC6 are found. These glycoproteins are the major macromolecular component of mucus and are also known to be regulated by PKA (47). Mucin gene expression varies with differentiation, inflammation and carcinogenesis (48), processes known to affect WT1 gene expression or subcellular localization. Recent observations have shown increased expression of MUC2, MUC5AC and MUC5B in association with secretory cell hyperplasia and metaplasia in the airways and fostered our interest in the processes controlling mucin secretion. Genomic proximity and detection of binding sites of WT1 in the promoter of mucins (detected by TESS, see methods), make these genes attractive targets to assess for WT1 de-repression.

Most of the literature regarding the transcription factor WT1 has focused on its role in cancer as WT1 is overexpressed in numerous solid tumors. It would be interesting to determine the role of NO in relation to WT1 function and oncogenesis. Our data provides new directions for research in cancer, inflammation and allergy and the role of NO in the expression of several genes.

6. References

1. Atkinson, J.J., and R.M. Senior. 2003. Matrix metalloproteinase-9 in lung remodeling. *Am J Respir Cell Mol Biol* 28:12-24.

2. Somerville, R.P., S.A. Oblander, and S.S. Apte. 2003. Matrix metalloproteinases: old dogs with new tricks. *Genome Biol* 4:216.

3. Han, Z., Junxu, and N. Zhong. 2003. Expression of matrix metalloproteinases MMP-9 within the airways in asthma. *Respir Med* 97:563-567.

4. Mautino, G., N. Oliver, P. Chanez, J. Bousquet, and F. Capony. 1997. Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid and by alveolar macrophages of asthmatics. *Am J Respir Cell Mol Biol* 17:583-591.

5. Lee, Y.C., H.B. Lee, Y.K. Rhee, and C.H. Song. 2001. The involvement of matrix metalloproteinase-9 in airway inflammation of patients with acute asthma. *Clin Exp Allergy* 31:1623-1630.

6. Kelly, E.A., W.W. Busse, and N.N. Jarjour. 2000. Increased matrix metalloproteinase-9 in the airway after allergen challenge. *Am J Respir Crit Care Med* 162:1157-1161.

7. McMillan, S.J., J. Kearley, J.D. Campbell, X.W. Zhu, K.Y. Larbi, J.M. Shipley, R.M. Senior, S. Nourshargh, and C.M. Lloyd. 2004. Matrix metalloproteinase-9 deficiency results in enhanced allergen-induced airway inflammation. *J Immunol* 172:2586-2594.

8. d'Ortho, M.P., C. Clerici, P.M. Yao, C. Delacourt, C. Delclaux, M.L. Franco-Montoya, A. Harf, and C. Lafuma. 1997. Alveolar epithelial cells in vitro

produce gelatinases and tissue inhibitor of matrix metalloproteinase-2. *Am J Physiol* 273:L663-675.

9. Hozumi, A., Y. Nishimura, T. Nishiuma, Y. Kotani, and M. Yokoyama. 2001. Induction of MMP-9 in normal human bronchial epithelial cells by TNF-alpha via NF-kappa B-mediated pathway. *Am J Physiol Lung Cell Mol Physiol* 281:L1444-1452.

10. Ichiyasu, H., J.M. McCormack, K.M. McCarthy, D. Dombkowski, F.I. Preffer, and E.E. Schneeberger. 2004. Matrix metalloproteinase-9-deficient dendritic cells have impaired migration through tracheal epithelial tight junctions. *Am J Respir Cell Mol Biol* 30:761-770.

11. Vermaelen, K.Y., D. Cataldo, K. Tournoy, T. Maes, A. Dhulst, R. Louis, J.M. Foidart, A. Noel, and R. Pauwels. 2003. Matrix metalloproteinase-9-mediated dendritic cell recruitment into the airways is a critical step in a mouse model of asthma. *J Immunol* 171:1016-1022.

12. Cataldo, D.D., K.G. Tournoy, K. Vermaelen, C. Munaut, J.M. Foidart, R. Louis, A. Noel, and R.A. Pauwels. 2002. Matrix metalloproteinase-9 deficiency impairs cellular infiltration and bronchial hyperresponsiveness during allergen-induced airway inflammation. *Am J Pathol* 161:491-498.

13. Naoum, J.J., K.J. Woodside, S. Zhang, P.G. Rychahou, and G.C. Hunter. 2005. Effects of rapamycin on the arterial inflammatory response in atherosclerotic plaques in Apo-E knockout mice. *Transplant Proc* 37:1880-1884.

14. Egi, K., N.E. Conrad, J. Kwan, C. Schulze, R. Schulz, and S.M. Wildhirt. 2004. Inhibition of inducible nitric oxide synthase and superoxide production

reduces matrix metalloproteinase-9 activity and restores coronary vasomotor function in rat cardiac allografts. *Eur J Cardiothorac Surg* 26:262-269.

15. Gu, Z., M. Kaul, B. Yan, S.J. Kridel, J. Cui, A. Strongin, J.W. Smith, R.C. Liddington, and S.A. Lipton. 2002. S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science* 297:1186-1190.

16. Mayers, I., T. Hurst, A. Radomski, D. Johnson, S. Fricker, G. Bridger, B. Cameron, M. Darkes, and M.W. Radomski. 2003. Increased matrix metalloproteinase activity after canine cardiopulmonary bypass is suppressed by a nitric oxide scavenger. *J Thorac Cardiovasc Surg* 125:661-668.

17. Mayers, I., T. Hurst, L. Puttagunta, A. Radomski, T. Mycyk, G. Sawicki, D. Johnson, and M.W. Radomski. 2001. Cardiac surgery increases the activity of matrix metalloproteinases and nitric oxide synthase in human hearts. *J Thorac Cardiovasc Surg* 122:746-752.

18. Marcet-Palacios, M., K. Graham, C. Cass, A.D. Befus, I. Mayers, and M.W. Radomski. 2003. Nitric oxide and cyclic GMP increase the expression of matrix metalloproteinase-9 in vascular smooth muscle. *J Pharmacol Exp Ther* 307:429-436.

19. Takimoto, E., H.C. Champion, M. Li, S. Ren, E.R. Rodriguez, B. Tavazzi, G. Lazzarino, N. Paolocci, K.L. Gabrielson, Y. Wang, and D.A. Kass. 2005. Oxidant stress from nitric oxide synthase-3 uncoupling stimulates cardiac pathologic remodeling from chronic pressure load. *J Clin Invest* 115:1221-1231.

20. Chen, Y., S. Hozawa, S. Sawamura, S. Sato, N. Fukuyama, C. Tsuji, T. Mine, Y. Okada, R. Tamino, Y. Ogushi, and H. Nakazawa. 2005. Deficiency of inducible nitric oxide synthase exacerbates hepatic fibrosis in mice fed high-fat diet. *Biochem Biophys Res Commun* 326:45-51.

21. Joos, L., J.Q. He, M.B. Shepherdson, J.E. Connett, N.R. Anthonisen, P.D. Pare, and A.J. Sandford. 2002. The role of matrix metalloproteinase polymorphisms in the rate of decline in lung function. *Hum Mol Genet* 11:569-576.

22. Shimajiri, S., N. Arima, A. Tanimoto, Y. Murata, T. Hamada, K.Y. Wang, and Y. Sasaguri. 1999. Shortened microsatellite d(CA)21 sequence down-regulates promoter activity of matrix metalloproteinase 9 gene. *FEBS Lett* 455:70-74.

23. Mundlos, S., J. Pelletier, A. Darveau, M. Bachmann, A. Winterpacht, and B. Zabel. 1993. Nuclear localization of the protein encoded by the Wilms' tumor gene WT1 in embryonic and adult tissues. *Development* 119:1329-1341.

24. Silberstein, G.B., K. Van Horn, P. Strickland, C.T. Roberts, Jr., and C.W. Daniel. 1997. Altered expression of the WT1 wilms tumor suppressor gene in human breast cancer. *Proc Natl Acad Sci U S A* 94:8132-8137.

25. Lee, S.B., and D.A. Haber. 2001. Wilms tumor and the WT1 gene. *Exp Cell Res* 264:74-99.

26. Sakamoto, Y., M. Yoshida, K. Semba, and T. Hunter. 1997. Inhibition of the DNA-binding and transcriptional repression activity of the Wilms' tumor gene

product, WT1, by cAMP-dependent protein kinase-mediated phosphorylation of Ser-365 and Ser-393 in the zinc finger domain. *Oncogene* 15:2001-2012.

27. Li, Z., J. Ajdic, M. Eigenthaler, and X. Du. 2003. A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. *Blood* 101:4423-4429.

28. Forte, L.R., P.K. Thorne, S.L. Eber, W.J. Krause, R.H. Freeman, S.H. Francis, and J.D. Corbin. 1992. Stimulation of intestinal Cl- transport by heat-stable enterotoxin: activation of cAMP-dependent protein kinase by cGMP. *Am J Physiol* 263:C607-615.

29. Ruiz-Velasco, V., J. Zhong, J.R. Hume, and K.D. Keef. 1998. Modulation of Ca2+ channels by cyclic nucleotide cross activation of opposing protein kinases in rabbit portal vein. *Circ Res* 82:557-565.

30. Drummond, I.A., S.L. Madden, P. Rohwer-Nutter, G.I. Bell, V.P. Sukhatme, and F.J. Rauscher, 3rd. 1992. Repression of the insulin-like growth factor II gene by the Wilms tumor suppressor WT1. *Science* 257:674-678.

31. Madden, S.L., D.M. Cook, J.F. Morris, A. Gashler, V.P. Sukhatme, and F.J. Rauscher, III. 1991. Transcriptional repression mediated by the WT1 Wilms tumor gene product. *Science* 253:1550-1553.

32. Wang, Z.Y., S.L. Madden, T.F. Deuel, and F.J. Rauscher, 3rd. 1992. The Wilms' tumor gene product, WT1, represses transcription of the platelet-derived growth factor A-chain gene. *J Biol Chem* 267:21999-22002.

33. Gilchrist, M., S.D. McCauley, and A.D. Befus. 2004. Expression, localization, and regulation of NOS in human mast cell lines: effects on leukotriene production. *Blood* 104:462-469.

34. Bickmore, W.A., K. Oghene, M.H. Little, A. Seawright, V. van Heyningen, and N.D. Hastie. 1992. Modulation of DNA binding specificity by alternative splicing of the Wilms tumor wt1 gene transcript. *Science* 257:235-237.

35. Mohamed, A.J., L. Vargas, B.F. Nore, C.M. Backesjo, B. Christensson, and C.I. Smith. 2000. Nucleocytoplasmic shuttling of Bruton's tyrosine kinase. *J Biol Chem* 275:40614-40619.

36. Ulanova, M., L. Puttagunta, M. Marcet-Palacios, M. Duszyk, U. Steinhoff, F. Duta, M.K. Kim, Z.K. Indik, A.D. Schreiber, and A.D. Befus. 2005. Syk tyrosine kinase participates in beta1-integrin signaling and inflammatory responses in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 288:L497-507.

37. Luecke, H.F., and K.R. Yamamoto. 2005. The glucocorticoid receptor blocks P-TEFb recruitment by NFkappaB to effect promoter-specific transcriptional repression. *Genes Dev* 19:1116-1127.

38. Boffelli, D., C.V. Weer, L. Weng, K.D. Lewis, M.I. Shoukry, L. Pachter, D.N. Keys, and E.M. Rubin. 2004. Intraspecies sequence comparisons for annotating genomes. *Genome Res* 14:2406-2411.

39. Scholz, H., and K.M. Kirschner. 2005. A role for the Wilms' tumor protein WT1 in organ development. *Physiology (Bethesda)* 20:54-59.

40. Rundlof, A.K., M. Carlsten, and E.S. Arner. 2001. The core promoter of human thioredoxin reductase 1: cloning, transcriptional activity, and Oct-1, Sp1, and Sp3 binding reveal a housekeeping-type promoter for the AU-rich element-regulated gene. *J Biol Chem* 276:30542-30551.

41. Choi, J.H., K.C. Choi, N. Auersperg, and P.C. Leung. 2006. Gonadotropins activate proteolysis and increase invasion through protein kinase A and phosphatidylinositol 3-kinase pathways in human epithelial ovarian cancer cells. *Cancer Res* 66:3912-3920.

42. Ye, Y., B. Raychaudhuri, A. Gurney, C.E. Campbell, and B.R. Williams. 1996. Regulation of WT1 by phosphorylation: inhibition of DNA binding, alteration of transcriptional activity and cellular translocation. *Embo J* 15:5606-5615.

43. Wang, Z.Y., Q.Q. Qiu, J. Huang, M. Gurrieri, and T.F. Deuel. 1995. Products of alternatively spliced transcripts of the Wilms' tumor suppressor gene, wt1, have altered DNA binding specificity and regulate transcription in different ways. *Oncogene* 10:415-422.

44. Himelstein, B.P., E.J. Lee, H. Sato, M. Seiki, and R.J. Muschel. 1998. Tumor cell contact mediated transcriptional activation of the fibroblast matrix metalloproteinase-9 gene: involvement of multiple transcription factors including Ets and an alternating purine-pyrimidine repeat. *Clin Exp Metastasis* 16:169-177.

45. Wu, T., T. Ikezono, C.W. Angus, and J.H. Shelhamer. 1994. Characterization of the promoter for the human 85 kDa cytosolic phospholipase A2 gene. *Nucleic Acids Res* 22:5093-5098.

46. Yan, C., H. Wang, B. Aggarwal, and D.D. Boyd. 2004. A novel homologous recombination system to study 92 kDa type IV collagenase transcription demonstrates that the NF-kappaB motif drives the transition from a repressed to an activated state of gene expression. *Faseb J* 18:540-541.

47. Bradbury, N.A. 2000. Protein kinase-A-mediated secretion of mucin from human colonic epithelial cells. *J Cell Physiol* 185:408-415.

48. Van Seuningen, I., P. Pigny, M. Perrais, N. Porchet, and J.P. Aubert. 2001. Transcriptional regulation of the 11p15 mucin genes. Towards new biological tools in human therapy, in inflammatory diseases and cancer? *Front Biosci* 6:D1216-1234.

Chapter IV: WT1 mediates nitric oxide-dependent repression of lymphocyte proliferation

1. Title

Human lymphocytes express the transcriptional regulator Wilms tumor 1 and it mediates nitric oxide-dependent repression of lymphocyte proliferation

2. Introduction

Nitric oxide (NO) functions as a powerful inhibitor of T-cell immunity both in vitro (1, 2) and in vivo (3, 4), in part by acting as an inhibitor of lymphocyte proliferation

(5, 6) through an apoptosis-independent mechanism (7). Interestingly, soluble guanylate cyclase (sGC) activity is critical (8) in these pathways. However the mechanisms involved have not been fully identified.

We have recently identified a critical downstream target of NO and sGC called Wilms tumor 1 (WT1). WT1 is a transcription factor that plays a critical role in differentiation and embryogenesis (9). In recent years WT1 has been identified in a number of adult epithelial tissues such as lung, uterine, testicular, breast, olfactory and renal epithelial cells (10). Additionally WT1 expression has been identified in leukocyte progenitors in bone marrow, indicating that WT1 may be critical in adult cells undergoing differentiation and proliferation.

Because WT1 is expressed by cells undergoing proliferation and it is regulated by NO and sGC, we postulated that human peripheral blood lymphocytes express WT1 and that WT1 activity is regulated by NO. In this study we report that adult human lymphocytes express WT1 mRNA and protein. We studied the localization and protein levels of WT1 in lymphocytes and identified that the NO-dependent inhibition of lymphocyte proliferation is associated with decreased expression of WT1.

3. Materials and methods

Antibodies: Monoclonal antibody against WT1 was obtained from Santa Cruz Biotechnologies, Inc. (F-6) (Santa Cruz, CA). Other antibodies included: peroxidase-conjugated goat anti-mouse IgG (BD Biosciences. Mississauga, ON Canada), donkey anti-mouse IgG, IRDye 800 conjugated (Rockland Immunochemicals, Inc, Gilbertsville, PA), mouse IgG1 isotype control (R&D Systems, Minneapolis),

peroxidase-conjugated affinity purified F(ab')2 fragment goat anti-mouse IgG (H + L) (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) and Rhodamine Red-X goat anti-mouse IgG.

T cell isolation: One hundred ml of blood was collected from peripheral vein of healthy donors in 10 ml heparinised collection tubes (Vacutainer™, BD Canada, Oakville, ON, Canada) and layered on Ficoll-Paque™ (Amersham Bioscience, Uppsala, Sweden). The mononuclear cell fraction was collected and incubated for 2 hr in RPMI 1640 supplemented with 10% FBS (Invitrogen Canada Inc., Burlington, Ontario, Canada) to allow adhesion of monocytes. Non-adhering cells where recovered and added to a nylon wool column (ZeptoMetrix Corp, Buffalo, NY, USA) to allow B cell adhesion. After elution of the column with RPMI 1640, the T cell enriched fraction was recovered. Using flow cytometry with anti-human-CD3-PERCP monoclonal antibody (BD Bioscience, Mississauga, ON. Canada) 85-90% of the cells were identified as T cells. The T cell enriched fraction was then incubated in RPMI 1640 supplemented with 10% FBS and phytohemagglutinin (PHA) for 72 hr to induce T cell proliferation. Cells were subsequently harvested for detection of WT1 and flow cytometry.

Flow cytometry: For the detection of intracellular WT1, 10 ml of whole blood was drawn from healthy donors in heparinised collection tubes (Vacutainer™, BD Canada). Blood was mixed with 6% dextran to allow sedimentation of red blood cells. The leukocyte fraction was collected and cells were fixed using 4% paraformaldehyde (PBS, 0.54% sucrose) and permeabilised with saponin 0.5%/PBS.

Non-specific binding was blocked by pretreatment with normal goat serum. After blocking, anti-human WT1 (2μg/ml) or mouse IgG isotype control (R&D Systems, Minneapolis) were added to the cell suspension and detection was done using an anti-mouse IgG labelled with R-Phycoerythrin (BD Bioscience). Detection of WT1 was done using BD FACSCalibur™ Flow Cytometer (BD Bioscience).

Reverse Transcription Polymerase Chain Reaction (RT-PCR): The technique was performed as previously described (11). WT1 primers were designed to span from exon 4 to exon 6, thus detecting the presence or absence of exon 5. The primer sequences used were developed based on the WT1 sequence of accession number NM_024426. The forward primer sequence was 5' GAT GAA CTT AGG AGC CAC CTT AAA 3' and the reverse primer sequence was 5' TAT GTC TCC TTT GGT GTC TTT TGA 3', which generated 412 (with exon 5) and 361 bp (without exon 5) long PCR products. Beta-actin (β-actin) was used as the internal positive control. β-actin primers were forward 5' GGCATC CTC ACC CTG AAG TA 3' and reverse 5' AGG GCA TAC CCC TCG TAG AT 3' generated from the sequence NM_001101. These primers amplified a 325 bp PCR product. Primers were analyzed using the BLAST sequencing program at Genbank to ensure unique complementation of *Homo sapiens*. PCR amplification was performed in cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, and a final cycle of 72°C for 10 min to complete polymerization. The number of cycles was optimized to be in the exponential phase of the reaction by performing the reaction at different cycles. Densitometric analysis of the gels was performed to select optimal PCR cycle numbers. WT1 and β-actin were amplified for

30 and 25 cycles respectively. PCR products were analyzed on a 2% agarose gel containing ethidium bromide and visualized under UV light.

The amplified PCR products were cloned into pCR2.1 plasmid vector using the T/A cloning kit (Invitrogen Life Technologies) as previously described (12). Double-stranded DNA sequencing was conducted using an ABI 373A automated sequencer (Applied Biosystems, Foster City, CA) and sequences were tested for homology using the BLAST sequencing program at Genbank.

Western blot: Cells ($2x10^6$) were harvested and homogenized with 0.5 ml lysis buffer (10 mM Hepes, 2 mM $MgCl_2$, 15 mM KCl, 0.1 mM EDTA, and 0.15% Nonidet P-40, containing a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) to a final concentration of 1 μg/ml. Samples were subjected to 7% SDS-polyacrylamide gel electrophoresis followed by blotting and immunodetection with antibodies using the Odyssey Imaging System (Li-cor Biosciences, Nebraska, USA).

Confocal microscopy: T cells were grown and treated as stated above. Cells were spun onto slides using the cytospin method as previously described (13). After permeabilization with 0.2% Triton X100, nonspecific binding was blocked with 10% FBS plus 3% BSA. Cells were incubated overnight at 4°C with monoclonal antibodies diluted 1 μg/ml in 50 μl of blocking buffer, washed three times with PBS and incubated with Rhodamine Red-X -conjugated secondary antibody for 2 hr at room temperature. DAPI was used as a nuclear marker. Coverslips were mounted on glass slides and cells were examined with a confocal microscope (Olympus Fluoview, FV1000, Olympus America Inc, Hauppauge, NY).

Statistics: Results are means ± S.E. of at least three independent experiments. They were analyzed using one-way analysis of variance, and when significant differences were found, the multiple comparison Tukey-Kramer test was used (GraphPad InStat). Values where $P \leq 0.05$ were considered statistically significant. The n values referred in figure legends represent independent experiments of the same cell culture.

4. Results

Blood mononuclear cells but not granulocytes express WT1.

Intracellular detection of WT1 in different human blood leukocyte populations was assessed by flow cytometry (Fig. 4.1). We observed that lymphocytes (Fig. 4.1B) and monocytes (Fig. 4.1C) expressed detectable levels of intracellular WT1 protein. However, granulocytes (Fig. 4.1D) showed little WT1.

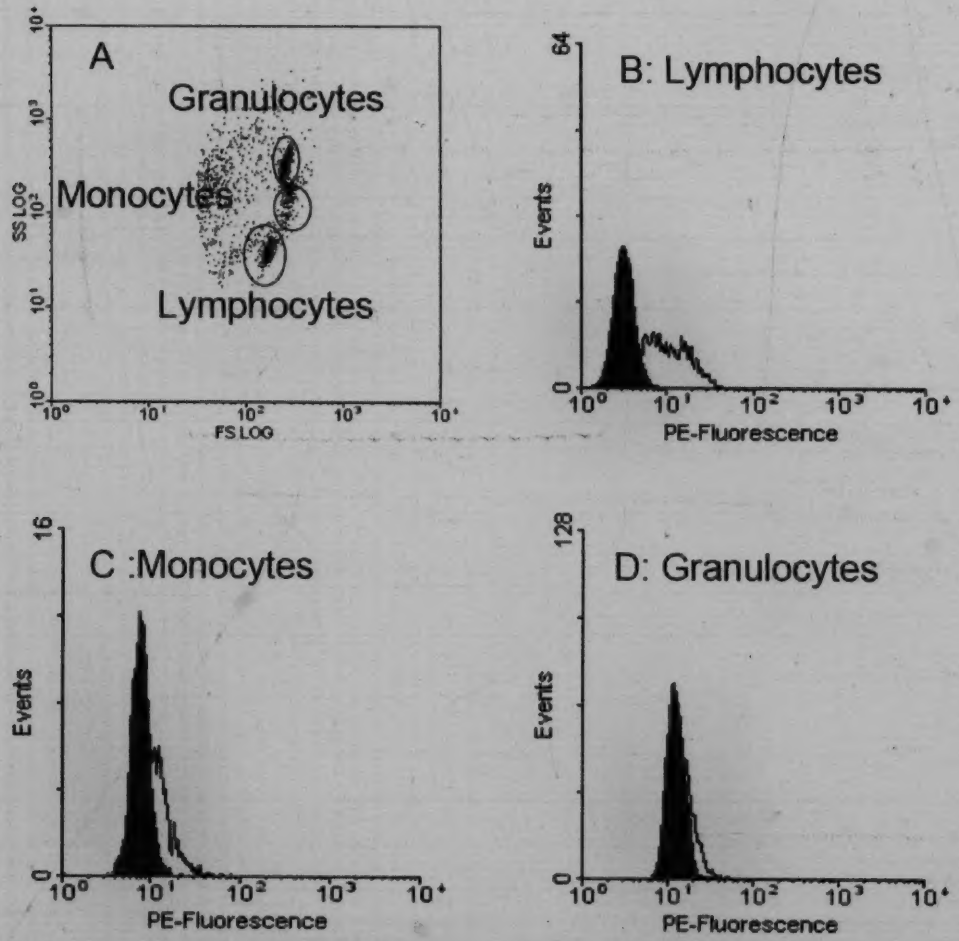


Figure 4.1: Flow cytometry of peripheral blood leucocytes showed expression of WT1 in lymphocytes and monocytes but not in granulocytes. Dot-Plot A depicts Side Scatter and Forward Scatter characteristics of whole blood allowing discrimination between lymphocytes, monocytes and granulocytes. Specific staining for WT1 was demonstrated by a shift to the right of fluorescence intensity detected in the FL2 fluorescence channel (575nm) detected in histogram B, C and D for different leukocytes populations.

Activated T Lymphocytes express increased levels of WT1.

Although B cells do not express WT1 (14), T cells express WT1 at least at the mRNA level (15). Proliferation of nylon wool enriched human T lymphocytes was induced by PHA for 72 hr. WT1 mRNA levels were significantly upregulated in PHA treated cells as compared with sham-treated cells (Fig. 4.2A). These treatments did not change levels of the internal loading control β-actin. The human LEC line A549 was used as a positive control for WT1 mRNA expression. WT1 mRNA levels in untreated A549 were significantly higher than in resting T lymphocytes, but not significantly different from PHA-treated cells. Equivalent cell numbers (2×10^6) were used to extract total RNA and prior to PCR, cDNA concentrations were adjusted to 50 ng/μl. WT1 expression significantly increases in T cells after 72 hr proliferation (Fig. 4.2B). The mean fluorescence intensity (MFI) of intracellular WT1 increased 2.4 fold after 72 hr of PHA stimulation ($p < 0.01$, $n = 3$). The increased expression of WT1 in proliferating T cells was also reflected by an increase of WT1 positive cells from 22±2% to 50±5% following PHA treated cells (figure 4.2D, $p < 0.01$, $n = 3$) compared to untreated T cells.

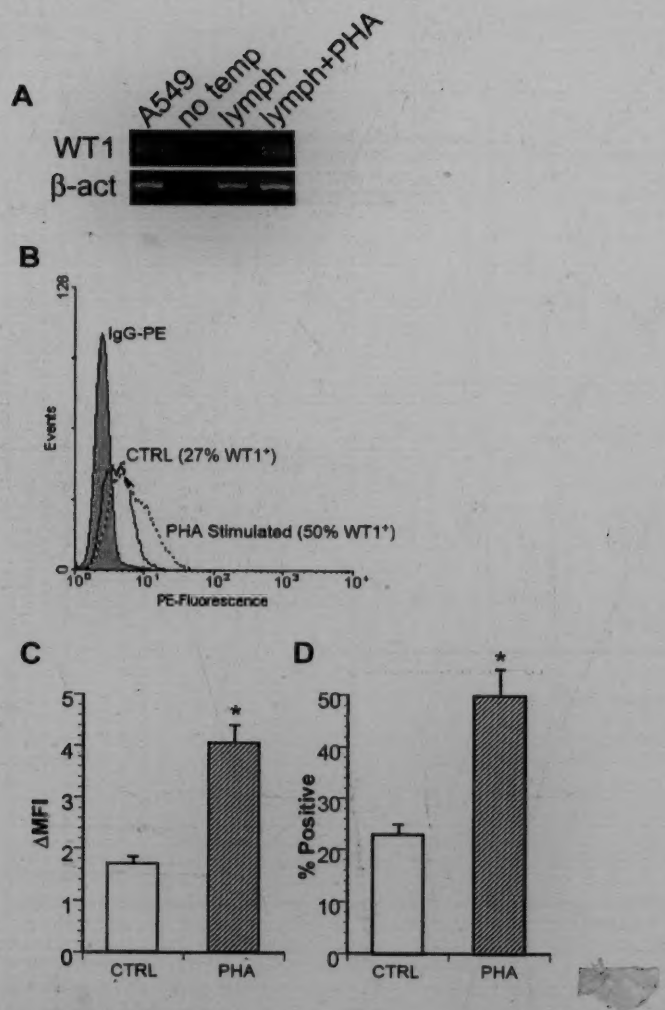


Figure 4.2: PHA-stimulated proliferating T cells express increased levels of WT1. (A) RT-PCR analysis of WT1 mRNA expression in human lung epithelial cells (A549), no template control (no temp), sham-treated lymphocytes (lymph) and PHA-treated (72 hr) lymphocytes (lymph+PHA). The loading control β-actin (β-act) was included. Equivalent number of cells was used to extract total RNA ($2x10^6$). DNA concentrations were adjusted to 50 ng/μl, prior to PCR. (B) Typical flow cytometry histogram depicting specific staining for WT1 demonstrated by a shift to the right of fluorescence intensity detected in the FL2 fluorescence channel (575nm) of PHA

treated T cells compared to untreated T cells and IgG isotype control. (C) Mean fluorescence intensity shift (ΔMFI) of WTI staining increase between PHA treated and untreated ($n = 3$, $p < 0.01$). D; Percentage of WT1 positive T cells increased after PHA treatment ($n = 3$). Statistical significance: $p < 0.05$ (*).

PHA-induced WT1 upregulation is repressed by NO.

WT1 protein expression in T cells was significantly upregulated by PHA treatment (72 hr) (Fig. 4.3A and 4.3B). Moreover, PHA-induced WT1 upregulation was inhibited in a concentration dependent manner with the NO donor SNOG. Confocal microscopy revealed that WT1 colocalized to the nucleus of PHA-treated T cells (Fig. 4.3C). Co-treatment with 300 μM SNOG for 72 hr did not change WT1 subcellular localization however, SNOG significantly reduced WT1 expression.

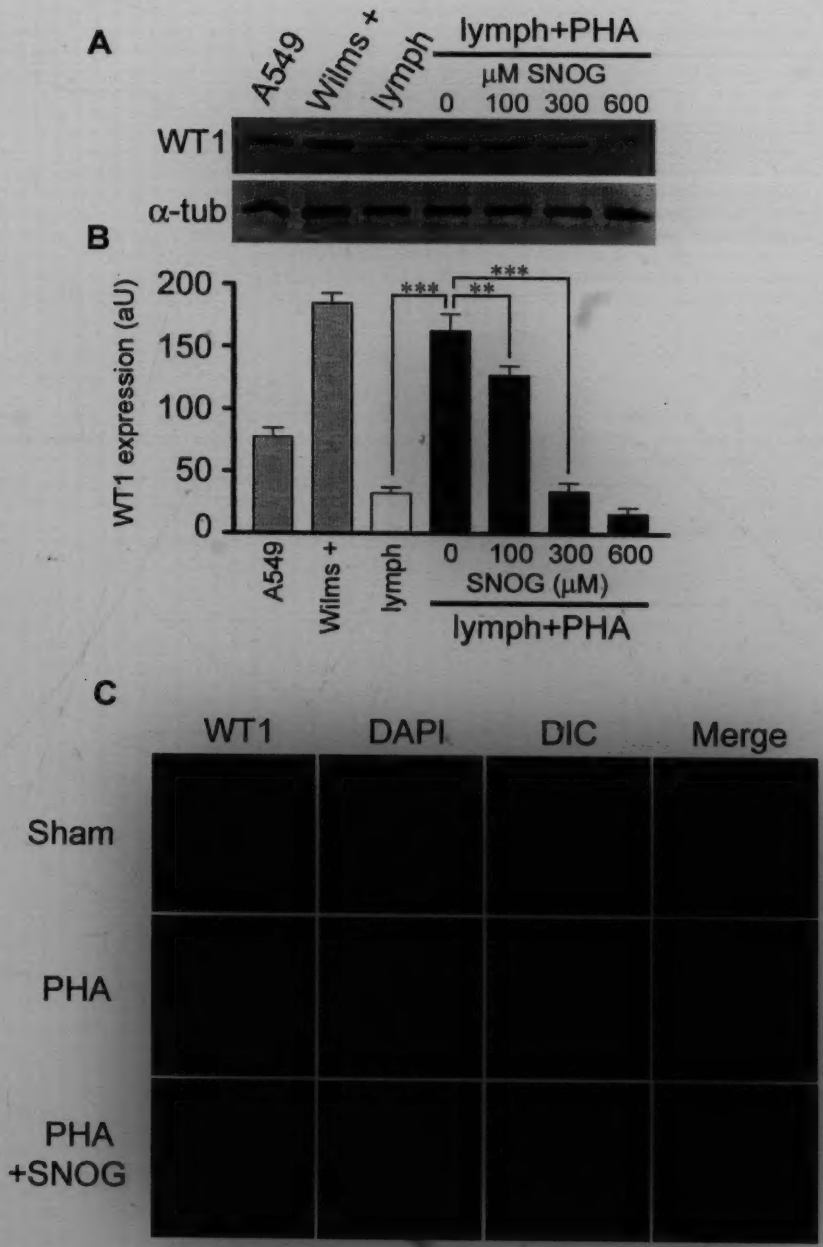


Figure 4.3: NO inhibits PHA-induced upregulation of WT1. (A) Western blot analysis for WT1 expression in human lung epithelial cells (A549), Wilms tumor positive control (Wilms+), untreated lymphocytes (lymph) and PHA-treated

lymphocytes stimulated with 0-600μM SNOG for 72 hr. (B) densitometric analysis of 3 independent experiments such as the representative experiment showed in A. (C) Confocal microscopy analysis demonstrating WT1 expression and localization in untreated lymphocytes (Sham), stimulated with PHA (PHA), and co-treated with 300 μM SNOG (n = 3). Statistical significance: $p < 0.01$ (**); $p < 0.001$ (***).

5. Discussion

We provide evidence that human T cells express WT1 mRNA and protein. NO has been shown to inhibit T cell proliferation and treatment with SNOG correlated with a decrease of WT1 levels, indicating a potential role for WT1 in the regulation of T cell biology.

WT1 expression has been extensively studied in many cell types both during development and in adult tissue. In agreement with our findings it has been reported that granulocytes differentiated *in vitro* from CD34+ progenitor cells do not express WT1. However, the same study reported that undifferentiated CD34+ cells express WT1 and the expression levels are downregulated in later stages of differentiation (16). However, very little is known about WT1 expression in T cells. Mitsuya et al, reported using RT-PCR WT1 expression in normal T lymphocytes (15) however this findings were not confirmed at the protein expression level. Interestingly, B cells do not express WT1 (14) suggesting that WT1 could be a NO-specific target in T cells. Our results confirm this observation and provide additional understanding in how WT1 might be regulated in T cells. This novel NO-dependent downregulation of WT1 expression in T cells might be a critical mechanism by which macrophage-derived NO regulates T cell proliferation by controlling the expression of

transcription factors like WT1. Our findings provide evidence for the WT1-mediated regulatory roles of NO in lymphocyte biology. It will be critical to determine whether WT1 targets selected gene regulation in association with modulation of T cell proliferation cycle.

6. References

1. Tomioka, H., and H. Saito. 1992. Characterization of immunosuppressive functions of murine peritoneal macrophages induced with various agents. *J Leukoc Biol* 51:24-31.
2. Mills, C.D. 1991. Molecular basis of "suppressor" macrophages. Arginine metabolism via the nitric oxide synthetase pathway. *J Immunol* 146:2719-2723.
3. Abrahamsohn, I.A., and R.L. Coffman. 1995. Cytokine and nitric oxide regulation of the immunosuppression in Trypanosoma cruzi infection. *J Immunol* 155:3955-3963.
4. Chung, C.S., G.Y. Song, W. Wang, I.H. Chaudry, and A. Ayala. 2000. Septic mucosal intraepithelial lymphoid immune suppression: role for nitric oxide not interleukin-10 or transforming growth factor-beta. *J Trauma* 48:807-812; discussion 812-803.
5. Albina, J.E., J.A. Abate, and W.L. Henry, Jr. 1991. Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation. Role of IFN-gamma in the induction of the nitric oxide-synthesizing pathway. *J Immunol* 147:144-148.

6. Krenger, W., G. Falzarano, J. Delmonte, Jr., K.M. Snyder, J.C. Byon, and J.L. Ferrara. 1996. Interferon-gamma suppresses T-cell proliferation to mitogen via the nitric oxide pathway during experimental acute graft-versus-host disease. *Blood* 88:1113-1121.

7. van der Veen, R.C., T.A. Dietlin, L. Pen, and J.D. Gray. 1999. Nitric oxide inhibits the proliferation of T-helper 1 and 2 lymphocytes without reduction in cytokine secretion. *Cell Immunol* 193:194-201.

8. Bingisser, R.M., P.A. Tilbrook, P.G. Holt, and U.R. Kees. 1998. Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. *J Immunol* 160:5729-5734.

9. Discenza, M.T., and J. Pelletier. 2004. Insights into the physiological role of WT1 from studies of genetically modified mice. *Physiol Genomics* 16:287-300.

10. Menke, A.L., A.J. van der Eb, and A.G. Jochemsen. 1998. The Wilms' tumor 1 gene: oncogene or tumor suppressor gene? *Int Rev Cytol* 181:151-212.

11. Marcet-Palacios, M., K. Graham, C. Cass, A.D. Befus, I. Mayers, and M.W. Radomski. 2003. Nitric oxide and cyclic GMP increase the expression of matrix metalloproteinase-9 in vascular smooth muscle. *J Pharmacol Exp Ther* 307:429-436.

12. Gilchrist, M., S.D. McCauley, and A.D. Befus. 2004. Expression, localization, and regulation of NOS in human mast cell lines: effects on leukotriene production. *Blood* 104:462-469.

13. Lacy, P., M.R. Logan, B. Bablitz, and R. Moqbel. 2001. Fusion protein vesicle-associated membrane protein 2 is implicated in IFN-gamma-induced piecemeal degranulation in human eosinophils from atopic individuals. *J Allergy Clin Immunol* 107:671-678.

14. Spinsanti, P., U. de Grazia, A. Faggioni, L. Frati, A. Calogero, and G. Ragona. 2000. Wilms' tumor gene expression by normal and malignant human B lymphocytes. *Leuk Lymphoma* 38:611-619.

15. Mitsuya, K., H. Sui, M. Meguro, H. Kugoh, Y. Jinno, N. Niikawa, and M. Oshimura. 1997. Paternal expression of WT1 in human fibroblasts and lymphocytes. *Hum Mol Genet* 6:2243-2246.

16. Maurer, U., J. Brieger, E. Weidmann, P.S. Mitrou, D. Hoelzer, and L. Bergmann. 1997. The Wilms' tumor gene is expressed in a subset of CD34+ progenitors and downregulated early in the course of differentiation in vitro. *Exp Hematol* 25:945-950.

Chapter V: General Discussion and Conclusions

1. Summary of major findings

We generated a septic shock model to study potential cross-talks between COX-2, iNOS and MMP-9 in rat VSM. Using a cocktail comprising LPS, IFNγ and PMA we induced co-expression of these three enzymes thus facilitating the study of their cross-talk. We found that iNOS inhibitors such as L-NAME and 1400W had a negative effect on MMP-9 upregulation when rat VSM cells were treated prior to stimulation with this cocktail. This observation led us to investigate this pathway to dissect the mechanism behind this cross-talk.

In subsequent experiments we determined that this pathway was not sensitive to SOD-mimetic compounds thus independent of the production of O_2^- and $ONOO^-$. However, this pathway was sensitive to the sGC inhibitor ODQ. Moreover, a membrane soluble cGMP analogue, recovered the cocktail-induced MMP-9 expression in the presence of ODQ, in rat VSM.

The sGC role in this pathway was also confirmed in human LEC. These cells were treated with TNF to stimulate the expression of iNOS and MMP-9. Like rat VSM, human LEC also exhibit a decrease of MMP-9 expression at the mRNA and enzyme levels when co-stimulated with ODQ. PKA and not PKG inhibitors blocked the TNF-

dependent upregulation of MMP-9. This observation led us to investigate the 5' flanking region of MMP-9 and propose WT1 as a potential PKA target involved in MMP-9 regulation.

Among other cell types, WT1 is expressed in adult mammary and kidney epithelial cells. Using a collection of molecular biology techniques we concluded that human LEC also express WT1. In resting conditions subcellular localization of WT1 is nuclear, however upon TNF stimulation WT1 translocates to the cytosol. TNF-dependent translocation of WT1 is iNOS-dependent since 1400W blocks this effect. Interestingly, WT1 knockdown results in a TNF-dependent induction of MMP-9 that loses its dependence on NO. This observation consolidated the model where NO de-represses the MMP-9 gene through a pathway involving WT1. Additionally, *in vivo* analysis of WT1 binding to the MMP-9 promoter shows a decrease of bound WT1 upon TNF treatment.

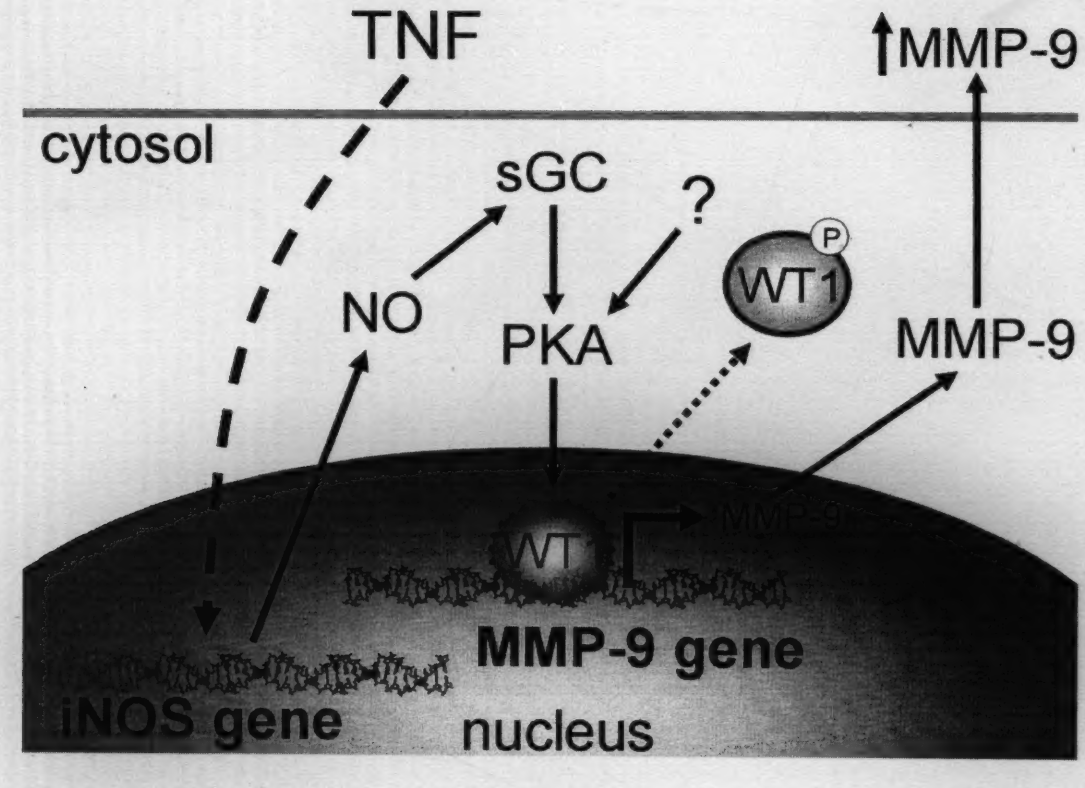


Figure 5.1: Thesis conceptual model.

2. Conceptual model

The MMP-9 promoter is repressed by WT1 in resting conditions. When LEC are exposed to cytokines or bacterial LPS, iNOS is upregulated resulting in increased levels of NO. A major NO target in human LEC is sGC. NO-dependent activation of sGC leads to production of cGMP, which among other mediators activates PKA. Activated PKA translocates to the nucleus and phosphorylates WT1. Phosphorylated WT1 will lose its DNA binding efficiency and translocate to the cytosol. Thus WT1-dependent repression of the MMP-9 gene is removed. At this point MMP-9 promoter

elements successfully induce the formation of the initiation complex and the MMP-9 gene is transcribed.

3. Results and their relation to original aims/questions

1. To establish a septic shock model in rat VSM by inducing co-expression of COX-2, iNOS and MMP-9.

The primary aim of this work was to study the nature of the cross-talk among the proinflammatory mediators COX-2, MMP-9 and iNOS. Rat VSM cells were used to establish a septic shock model in which COX-2, MMP-9 and iNOS were co-expressed. We achieved this objective with a rat VSM cell line A7r5 stimulated with LPS, IFN-γ, and PMA. Although this cocktail of pro-inflammatory mediators was very aggressive, it effectively engaged pathways to stimulate production of COX-2, iNOS and MMP-9 (Fig. 1.6).

2. To study the interactions between MMP-9, iNOS and COX-2 in rat VSM.

NO was a strong candidate to participate in the regulation of MMP-9 in rat VSM and human LEC. As described in chapter 1, there was substantial evidence supporting this proposal, but there was no evidence suggesting a role of NO in the transcriptional regulation of MMP-9.

In the pilot study described in chapter 2, we concluded that NO was a major regulator of MMP-9 transcription and the pathway was found to function through the activation of sGC. The latter is one of the most studied mediators of NO biology stimulating the conversion of GTP to cGMP.

3. To determine if the interactions apply to human LEC.

The pathway described in rat VSM cells was also present in human LEC. The observation provides more evidence on the relevance of NO functions as a regulator of MMP-9 gene expression. These findings also gave us the means to further dissect this pathway in a human-based system. In addition to findings in VSM, we determined that TNF-dependent upregulation of MMP-9 is mediated by PKA by downstream of sGC. *In silico* analysis of the MMP-9 promoter indicated that a PKA target and also a transcriptional repressor, WT1 could be a potential regulator of the MMP-9 gene in this pathway.

4. To characterize WT1 expression in human LEC.

Although WT1 expression in the lung had been demonstrated in rat and mouse, there was no report that human LEC could express this protein. Using a number of molecular biology approaches we concluded that at least 2 different WT1 transcripts are produced by LEC at the mRNA level and that a 54 kDa protein is expressed. Immunohistochemistry analysis of normal lungs demonstrated that WT1 was expressed in human ciliated LEC in particular within the nuclei. WT1 localization to the nucleus of human LEC in resting conditions was confirmed by confocal microscopy.

5. To determine the mechanisms by which WT1 regulates MMP-9 promoter.

WT1 expression was not changed by TNF treatment; however WT1 subcellular localization was altered in the presence of TNF. TNF induced WT1 translocation from the nucleus to the cytosol. Translocation was blocked by the iNOS inhibitor 1400W, indication that WT1 translocation was regulated by NO.

Using two independent molecular biology approaches (e.g. DNA-pulldown and ChiP) we demonstrated that WT1 binds to the MMP-9 promoter *in vivo*, at the predicted WT1 binding site (CA repeat). Binding was reduced in the presence of TNF in correlation with MMP-9 upregulation. WT1 knockdown by siRNA resulted in upregulation of MMP-9 protein/activity, even in the presence of the iNOS inhibitor 1400W.

WT1 expression and its regulation by NO have not been previously reported. Binding of WT1 to the CA repeat present in the MMP-9 promoter is also a novel finding. Although others have reported that PKA phosphorylates WT1 and this modification results in translocation of WT1 to the cytosol, the impact of such process on MMP-9 expression had not been studied. Validation of the role that PKA played was performed using the PKA inhibitor myr-PKA.

6. To study WT1 expression in leukocytes.

In light of the finding that WT1 function was regulated by NO we tested the hypothesis that WT1 would be a potential target of NO in lymphocytes. We confirmed that T cells are WT1 positive and that treatment with PHA induced proliferation in correlation with an increase of WT1 levels. The PHA-induced upregulation of WT1 was blocked in a concentration dependent manner by the NO donor SNOG. These data suggest that WT1 may be an important regulator of T cell proliferation and that the NO-dependent suppression of T cell proliferation could be mediated by WT1.

In chapter 3, we discovered that NO regulates WT1 subcellular localization through the activation of PKA and subsequent phosphorylation of WT1 in LEC. It is possible

that in lymphocytes NO-dependent down regulation of WT1 is due to an alternative pathway in which NO can affect WT1 mRNA half-life.

4. Results and their relation to the literature

NO-dependent upregulation of MMP-9 appears to be in contrast to the data reported by other laboratories. The cell type, stimuli and time of activation are some of the potential reasons for the contrasting data in the literature. The dual nature of NO is present in many systems, where NO concentrations may determine whether the effect is physiological or pathological. Given the multitude of pathways that are modulated by NO it is not surprising that it has so many effects on MMP-9. At higher concentrations NO and $ONOO^-$ might activate MMP-9 and reduce its mRNA half-life, whereas at lower concentrations it might inhibit NF-kB activation and also de-repress the MMP-9 gene by controlling WT1 function. In both cases, in high and in low concentrations, NO performs seemingly opposing effects. Thus NO duality is not explained by its concentration levels alone. Cell type is another important factor as exemplified by our own finding with NO affecting WT1 localization in LEC and protein levels in lymphocytes. Type of stimulation and treatment time are other important factors that might affect NO function.

WT1 is a resent mediator found to be regulated by NO. WT1 expression in human LEC is not surprising given the fact that WT1 had been identified in multiple other epithelial cells and found in rat and mouse lung. Additionally, we found WT1 expression in human lymphocytes. Similarly to epithelial cells, lymphocytes proliferate in response to stimuli like PHA. Although there is no conclusive mechanism by which WT1 is involved in cell cycle, WT1 overexpression in a number

of tumors and in morphogenesis hints towards a role in proliferation. As discussed in chapter 4, WT1 level reduction correlated with NO-dependent repression of lymphocyte proliferation offering a potential explanation for how high WT1 levels might cause or enhance tumorigenic processes.

Interestingly, in LEC NO regulates WT1 through a different pathway. In this sGC-mediated pathway, we found that PKA, not PKG was responsible for the signal transduction downstream of sGC. A number of reports have described this observation in the literature and it is thought that cGMP can also activate PKA (1-3). PKA activity is increased through the activation of AC (4). AC produces cAMP that is responsible for the activation of PKA. However, our findings and the reports referenced above, indicate that cGMP can also activate PKA. Pathways that activate adenylate cyclase and PKA such as adenosine (through adenosine receptors) and isoproterenol, epinephrine and norepinephrine (through adrenergic receptors) may also promote WT1 phosphorylation and enhance production of MMP-9 in the presence of appropriate stimuli such as TNF.

An alternative pathway supporting our observations involves the enzyme that regulates cAMP levels, phosphodiesterase (PDE). Levels of cAMP are regulated by degradation through hydrolysis of the PDE bond of 3'-5'-cyclic AMP to inactive 5′-AMP. In particular PDE3A is known to degrade cAMP, however PDE3A is inhibited by cGMP thus in conditions of high cGMP, cAMP levels increase. This cross-talk is responsible for the control of platelet activation (5). And it explains the sGC and PKA dependent observations described above.

5. Relevance/significance of findings

To our knowledge we are the first to report WT1 expression in human LEC. The exciting finding that NO is an important regulator of WT1 function may affect many other genes that also have a WT1 binding site such as nNOS, Syk, CFTR and the mucin family. Because NO and MMP-9 are over produced in many cancer types it would be interesting to determine the role of WT1 in these systems. WT1 itself is overexpressed in multiple cancers and it is possible that high NO levels signal to maintain the MMP-9 gene de-repressed.

MMP-9 is an aggressive enzyme and it is believed that its overexpression in the lungs results in tissue injury. Understanding the mechanism that provokes MMP-9 upregulation could lead to new opportunities for pharmacological intervention.

Collectively our results reflect the importance of structural tissues in inflammatory conditions. It is interesting to see that LEC-derived NO has important autocrine functions such as the upregulation and enzyme activation of other inflammatory mediators such as MMP-9.

More than two decades of research since the discovery of NO have provided us with significant insight into the mechanism underlining NO roles. Regulation of MMP-9 gene expression by NO adds MMP-9 to a growing list of genes regulated by NO. Although NO is still viewed as a potential pharmaceutical target for the treatment of asthma and septic shock, it is also important to realize that NO is involved in the regulation of many genes and undesirable effects are likely to arise as a consequence of NO multifaceted nature.

6. Future directions

It would be valuable to purify and sequence WT1 from human LEC. This data would conclusively demonstrate WT1 expression in LEC but also would provide the means to determining potential posttranscriptional modifications in WT1 such as sumoylation, phosphorylation and nitration/nitrosylation. Additionally, this method would provide sequence-based information of the different isoforms that could be co-expressed.

It would also be important to test our findings in human primary LEC. Although WT1 expression was tested in a number of LEC types including BEAS-2b (viral-immortalized) and primary LEC, the major findings presented in this thesis were tested in the ATII cell line A549, a tumor cell.

More detailed studies of the WT1-DNA binding also need to be carried out. ChiP is a powerful technique to study *in vivo* binding of putative transcription factors to their binding site and in conjugation with real time PCR this technique could provide more quantitative information with regards to WT1 binding. With this technique in place, the role of sGC, PKA and iNOS inhibitors could be studied in terms of WT1 binding to the MMP-9 promoter.

We have performed preliminary experiments to further evaluate the role of PKA in the phosphorylation of WT1 Ser-residues. To this end we used an anti-serine antibody rabbit polyclonal to phosphoserine (ab9334) from Abcam Cambridge, MA; and measured WT1-phosphorylation levels upon stimulation of A549 with TNF. We also measured the effect of 1400W to determine the NO dependency of the phosphorylation levels. We found that TNF induced a significant upregulation of

WT1 phosphorylation and incubation with 1400W prevented this upregulation (App. 5).

WT1 has been reported to bind mRNA potentially participating in post-transcriptional regulation. Interestingly, we have observed that the WT1 mRNA binding site (6) is present in the MMP-9 mRNA (App. 3) indicating that WT1 may regulate MMP-9 expression at multiple levels. It would be interesting to test this hypothesis which would enhance our understanding of the mechanisms used by WT1 to regulate protein expression.

7. References

1. Li, Z., J. Ajdic, M. Eigenthaler, and X. Du. 2003. A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. *Blood 101:4423.*

2. Forte, L. R., P. K. Thorne, S. L. Eber, W. J. Krause, R. H. Freeman, S. H. Francis, and J. D. Corbin. 1992. Stimulation of intestinal Cl- transport by heat-stable enterotoxin: activation of cAMP-dependent protein kinase by cGMP. *Am J Physiol 263:C607.*

3. Ruiz-Velasco, V., J. Zhong, J. R. Hume, and K. D. Keef. 1998. Modulation of Ca2+ channels by cyclic nucleotide cross activation of opposing protein kinases in rabbit portal vein. *Circ Res 82:557.*

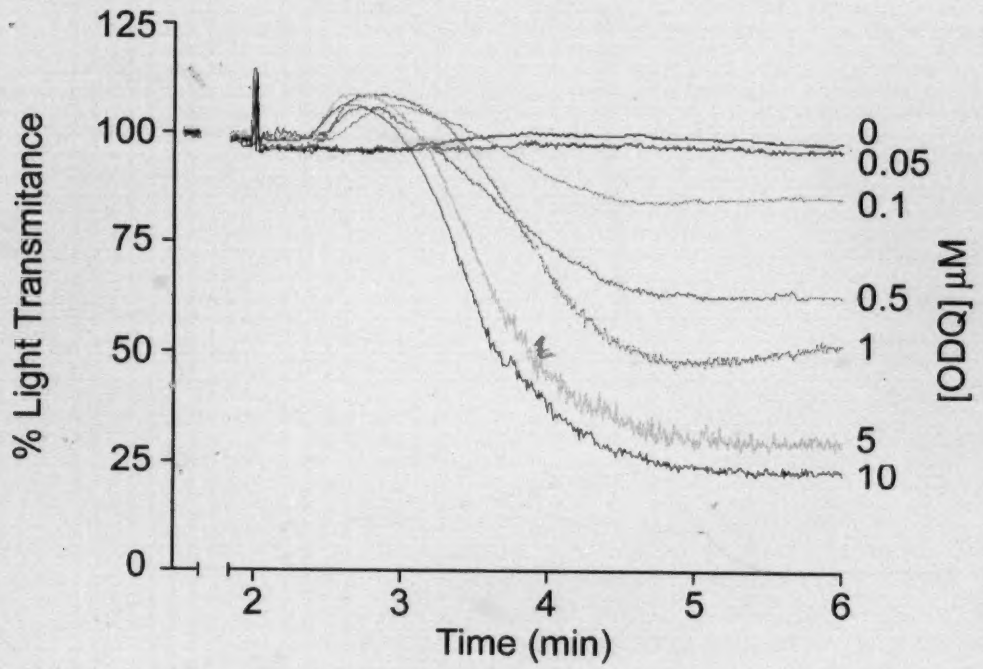
4. Robinson-White, A., and C. A. Stratakis. 2002. Protein kinase A signaling: "cross-talk" with other pathways in endocrine cells. *Ann N Y Acad Sci 968:256.*

5. Colman, R. W. 2004. Platelet cyclic adenosine monophosphate phosphodiesterases: targets for regulating platelet-related thrombosis. *Semin Thromb Hemost 30:451.*

6. Bardeesy, N., and J. Pelletier. 1998. Overlapping RNA and DNA binding domains of the wt1 tumor suppressor gene product. *Nucleic Acids Res 26:1784.*

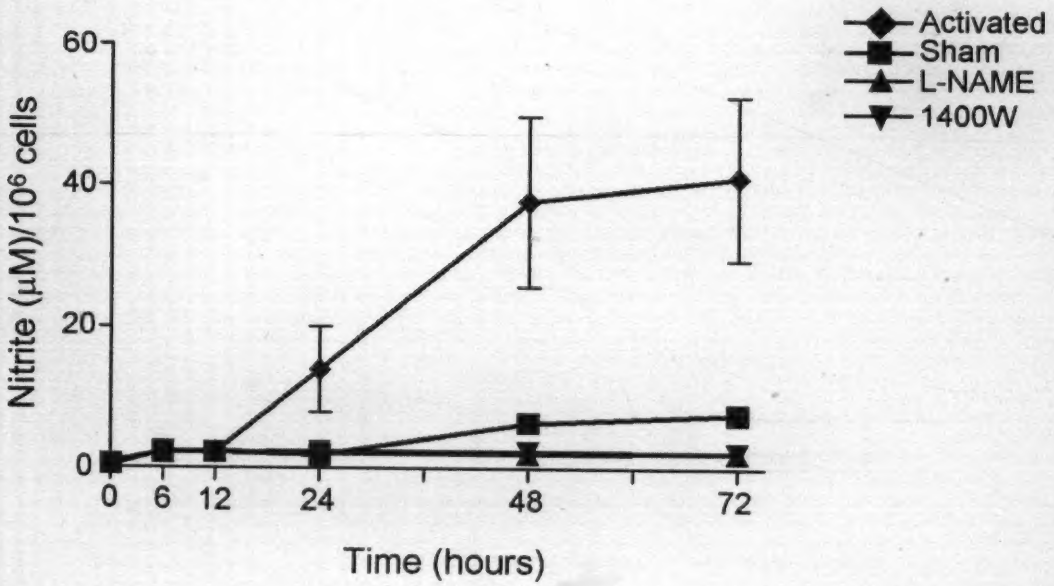
Appendix 1

ODQ biological activity measured by its effect on platelet aggregation. ODQ stimulates platelet aggregation in a concentration dependent manner. These experiments were perform to confirm ODQ biological activity.



Appendix 2

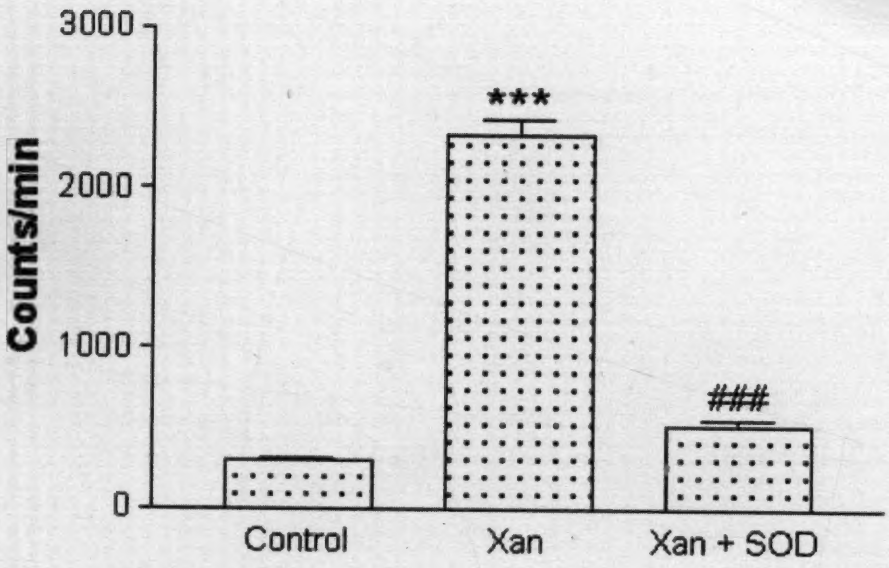
The Griess reaction was used to measure indirectly the NO released from VSM cells stimulated with the cocktail.



Appendix 3

SOD activity was tested using a superoxide generator, xanthine oxidase and the O_2^- levels were monitored by chemiluminescence (Fig 5-B) [25]. SOD was effective in reducing the levels of O_2^- to almost control levels at the concentration of 15 Units/ml, but the upregulation of MMP-9 mRNA was independent of $ONOO^-$. SOD was shown to effectively scavenge superoxide ion using a luminescence approach (n = 6). Statistical significance: P<0.05 (*), (#); P<0.001 (***), (###).

Fig 5-B



Appendix 4

WT1 RNA recognition motif is found in the MMP-9 mRNA at positions 1002-1021. This motif is shown in red.

agacaccucugcccucaccaugagccucuggcagccccuggucccuggugcuccuggugcugggcugcugcuu

ugcugccccagacagcgccaguccacccuugugcucuucccuggagaccugagaaccaaucucaccgacaggc

agcuggcagaggaauaccuguaccgcuaugguuacacucggguggcagagaugcguggagagucgaaaucuc

uggggccugcgcugcugcuucuccagaagcaacugucccugcccgagaccggugagcuggauagcgccacgcu

gaaggccaugcgaaccccacggugcggggucccagaccugggcagauuccaaaccuuugagggcgaccucaag

uggcaccaccacaacaucaccuauuggauccaaaacuacucggaagacuugccgcgggcggugauugacgacg

ccuuugcccgcgccuucgcacuguggagcgcggugacgccgcucaccuucacucgcguguacagccgggacgc

agacaucgucauccaguuuggugucgcggagcacggagacggguaucccuucgacgggaaggacgggcuccu

ggcacacgccuuuccuccuggccccggcauucagggagacgcccauuucgacgaugacgaguuguggucccug

ggcaagggcgucgugguuccaacucgguuuggaaacgcagauggcgcggccugccacuuccccuucaucuuc

gagggccgcuccuacucugccugcaccaccgacggucgcuccgacggcuugcccuggugcaguaccacggcca

acuacgacaccgacgaccgguuuggcuucugccccagcgagagacucuacacccaggacggcaaugcugaugg

gaaacccugccaguuuccauucaucuuccaaggccaauccuacuccgccugcaccacggacggucgcuccgacg

gcuaccgcuggugcgccaccaccgccaacuacgaccgggacaagcucuucggcu**ucugcccgacccgagcuga**

cucgacggugaugggggggcaacucggcgggggagcugugcgucuuccccuucacuuuccuggguaaggagua

cucgaccuguaccagcgagggccgcggagaugggcgccucuggugcgcuaccaccucgaacuuugacagcgac

aagaaguggggcuucugcccggaccaaggauacaguuuguuccucguggcggcgcaugaguucggccacgcg

cugggcuuagaucauuccucagugccggaggcgcucauguacccuauguaccgcuucacugaggggccccccu

ugcauaaggacgacgugaauggcauccggcaccucuaugguccucgcccugaaccugagccacggccuccaac

caccaccacaccgcagcccacggcucccccgacggucugccccaccggacccccccacuguccaccccucagagcg

ccccacagcuggccccacaggucccccccucagcuggccccacaggucccccccacugcuggcccuucuacggcca

cuacugugccuuugaguccgguggacgaugccugcaacgugaacaucuucgacgccaucgcggagauuggga

accagcuguauuuguucaaggaugggaaguacuggcgauucucugagggcaggggagccggccgcagggcc

ccuuccuuaucgccgacaaguggcccgcgcugccccgcaagcuggacucggucuuugaggagcggcucuccaa

gaagcuuuucuucuucucugggcgccagguguggguguacacaggcgcgucggugcugggcccgaggcguc

uggacaagcugggccugggagccgacguggcccaggugaccggggcccuccggaguggcagggggaagaugc

ugcuguucagcgggcggcgccucuggagguucgacgugaaggcgcagaugguggaucccccggagcgccagcg

agguggaccggauguucccccggggugccuuuggacacgcacgacgucuuccaguaccgagagaaagccuauu

ucugccaggaccgcuucuacuggcgcgugaguucccggagugaguugaaccagguggaccaaguggggcuacg

ugaccuaugacauccugcagugcccugaggacuagggcucccguccugcuuuggcagugccauguaaaucccc

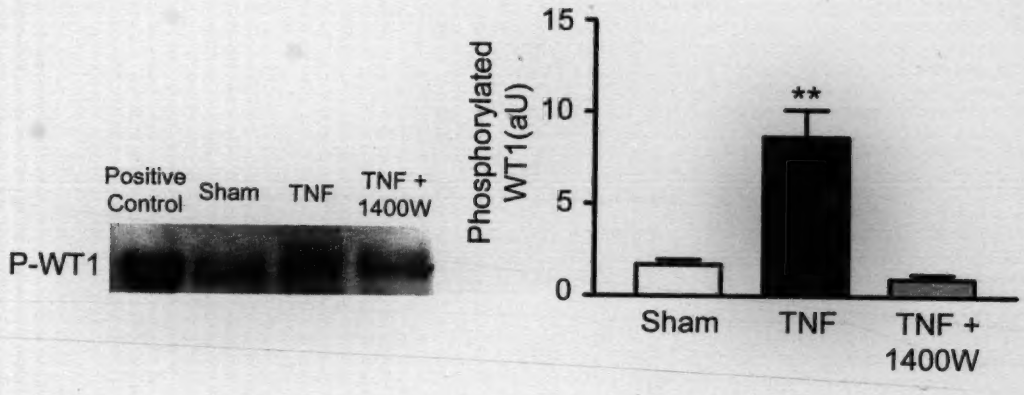
acugggaccaacccuggggaaggagccaguuugccggauacaaacugguauucuguucuggaggaaagggag

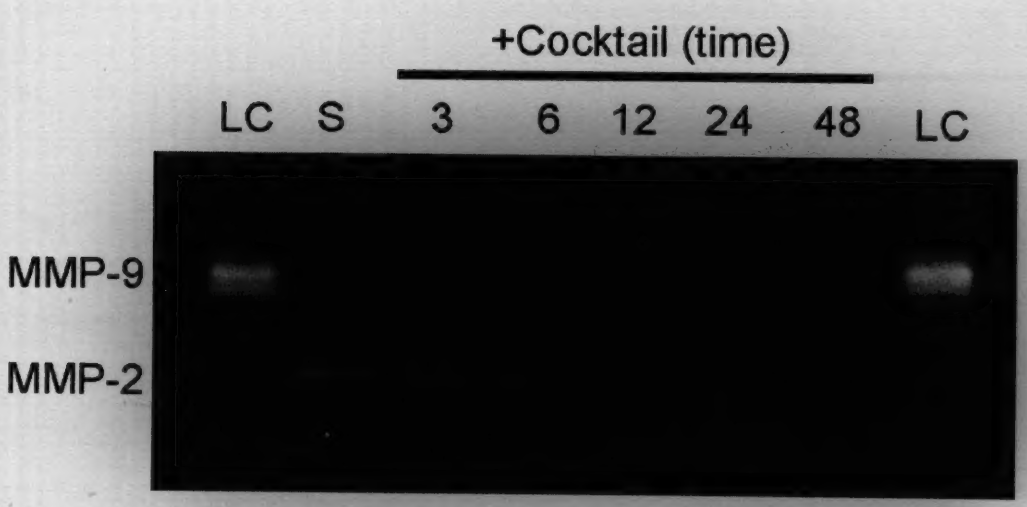
gaguggaggugggcugggcccucucuucucaccuuuguuuuuuguuggaguguuucuaauaaacuuggauu

cucuaaccuuuaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

Appendix 5

WT1 phosphorylation is NO-dependent. LEC line A549 was stimulated and immunoprecipitated with the anti-WT1 monoclonal antibody F6 (see chapter 3 for this methodology). Following immunoprecipitation the samples were analysed by western blot using an anti-Ser antibody (left panel). A positive control (human brain) was used to detect phosphorylation. TNF significantly induced WT1 Ser-phosphorylation (right panel) and this effect was blocked by the iNOS inhibitor 1400W.



Appendix 6

MMP-9 production from A7r5 cells was measured at 3, 6, 12, 24 and 48 hr after stimulation with a cocktail containing PMA, LPS and IFN-γ. The best time to study MMP-9 production was 12 hr. Loading controls (LC) are shown.